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APPLICATIONS OF LC/MS IN PHARMACEUTICAL ANALYSIS

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ABSTRACT

The power and versatility of liquid chromatography–mass spectrometry (LC/MS) and LC/MS/MS is discussed in terms of "successful practices," and how individual investigators can follow these practices to obtain useful information during different stages of the drug development process. This chapter focuses on various applications of LC and electrospray mass spectrometry for pharmaceutical analysis. The objective is to provide ideas and experimental designs for the solution of qualitative problems including drug impurity profiling, HPLC method development, deficit in drug mass balance, drug discoloration, and drug counterfeiting. Many examples are presented to illustrate how LC/MS and LC/MS/MS can be used to provide answers to these real-world problems.

I. INTRODUCTION

The year 2002 was an extraordinary year for liquid chromatography–mass spectrometry (LC/MS) practitioners. On October 9, 2002, the Royal Swedish Academy of Sciences announced their decision to award the Nobel Prize in Chemistry to John B. Fenn, Koichi Tanaka, and Kurt Wüthrich for their development of analytical methods for the identification and structural analysis of biological macromolecules. Fenn and Tanaka shared the prize for developing electrospray and soft-laser desorption, respectively. These soft-ionization techniques allow macromolecules to be ionized without fragmentation.

The application of electrospray ionization (ESI) to macromolecules was first described by Yamashita and Fenn[1] in 1984, and he later applied this technique to the study of proteins in 1988. In ESI, charged droplets of protein were produced and the solvent was stripped away, leaving only the free protein ion. In 1987, Tanaka demonstrated that laser pulses from a low-energy nitrogen laser could be used to ionize proteins from a surface. As described in a paper presented during the Second Japan–China Joint Symposium on Mass Spectrometry, proteins are desorbed from a glycerol matrix containing cobalt powder. The cobalt powder was necessary to increase absorption of the laser photons.[2]

The LC/MS combination has been practiced for many years. Various types of interfaces have been developed for LC/MS, including the fast atom bombardment (FAB) source by Barber et al.[3] in 1981 and thermospray by Blakely and Vestal[4] in 1983. However, a broad acceptance of

this hyphenated technique in the pharmaceutical industry did not occur until the advent of new ionization methods such as electrospray (ES), which was developed by Yamashita and Fenn[1] in the 1980s. Since then, LC/MS has become indispensable to the pharmaceutical industry. In addition to new ionization methods, the driving force behind the growth and success of LC/MS was its versatility in experimental designs, its extraordinary capability to generate both qualitative and quantitative information, as well as the reliability and affordability of commercially available systems. Although many applications became routine, even to the point of "walk-up" automated experiments (open access system and open access lab), other applications continued to demand the attention of highly skilled scientists for the purpose of designing experiments and interpreting data. Table 1 summarizes currently available mass spectrometers from major vendors that can be interfaced to liquid chromatographs.

LC/MS is notable for its diversity in instrumentation and applications. Although this chapter provides an overview of basic theory and instrumentation, the focus is on applications of LC combined with electrospray mass spectrometry in pharmaceutical analysis. Most types of new molecular (medicinal) entities (NMEs) evaluated for drug purposes can be analyzed using electrospray mass spectrometry, including medium polar to polar compounds, and ranging from low-molecular-weight amines or acids to much larger peptides and proteins. Figure 1 illustrates the compatibility of different compounds with various interfaces/ionization techniques.

Different mass analyzers may impose unique technical requirements when interfaced to LC. Understanding the operating principles and technical properties of both LC/MS interfaces and mass analyzers is deemed beneficial. A brief overview of the history of the development of LC/MS interfaces is given in Section II, which is followed in Section III by a summary of working principles and characteristics of commonly used mass analyzers.

Bringing a promising pharmaceutical candidate to the market can take as long as 10 years and cost $300–700 millions. Many different analytical techniques are employed to obtain necessary information during different developmental phases, from drug discovery to chemical/formulation development, clinical studies, stability evaluation, and quality control. LC/MS has a particularly wide range of applications in the pharmaceutical industry, where the quantitation and structural characterization/identification of thermally labile and hydrophilic compounds are routinely required—for example, structural proof and characterization of drug lead compounds, kinetic studies and impurity profiling at the various steps and scales of chemical synthesis, stability and impurity testing of pharmaceutical formulations and packaging components, toxicological testing of drug candidates in different dosing vehicles, and pharmacokinetic/metabolic investigations for pre-clinical and clinical

TABLE I Manufacturers of Mass Spectrometers Interfaceable to LC

Vendor	System name	Mass analyzer	Interface
Agilent	LC/MSD Trap	IT	API
http://www.chem	LC/MSD TOF	TOF	API
agilent.com			
Bruker Daltonics	Esquire 2000	IT	API
http://www.bdal.com	Esquire 3000 plus	IT	API
	EsquireHCT	IT	API
	BioTOF II	TOF	ESI
	BioTOF Q	Q-TOF	ESI
	Apex IV and Apex-Q	FTMS	Multiple inlets
IonSpec	HiResESI FTMS	FTMS	ESI
http://www.ionspec.com	Explorer FTMS		Multiple
JEOL	AccuTOF	TOF	API
http://www.jeol.com	LCmate	B	Multiple inlets
Waters/Micromass	Q-Tof API-US	Q-TOF	API
http://www.waters.com/	Q-Tof Ultima API	Q-TOF	API
WatersDivision	Q-Tof micro	Q-TOF	API
	Quattro micro API	QQQ	API
	Quattro Ultima Pt	QQQ	API
	Quattro Premier	QQQ	API
	LCT	TOF	API
Applied Biosystem	API 150EX LC/MS	Q	API
and Sciex	API 2000/3000/4000,	QQQ	API
http://www.appliedbiosy	LC/MS/MS		
stems.com	API QSTAR™ Pulsar i	Q-TOF	Multiple
	Hybrid LC/MS/MS		
	System 4000 Q TRAP™	Q-IT	API
	LC/MS/MS System		
Thermo Finnigan	LCQ Deca XP MAX	Ion-trap	API
http://www.thermo.com	LCQ Advantage MAX	IT	API
	MAT95XP-Trap	IT	API
	LTQ FT MS	FTMS	API
	TSQ QUANTUM	QQQ	API
	Surveyor MSQ	Q	API

studies. It is worth noting that the chosen analytical techniques continue to play significant roles even after the launch of the drug product in the market. LC/MS is one of the few tools which continues to play a decisive role throughout all of the product's life cycle stages.

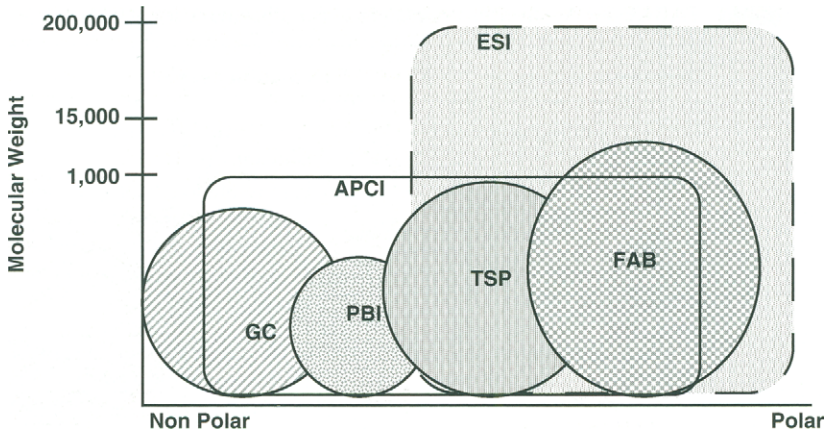


FIGURE 1 **Analyte compatibility with different interfaces/ionization techniques (courtesy of Thermo Finnigan).**

Like gas chromatography–mass spectrometry (GC/MS), integrating LC with MS brings together the potential of utilizing the intrinsic power of both LC and MS, thus expanding the analytical capabilities of both techniques. The importance of LC/MS in the pharmaceutical industry compared to GC/MS is the expansion of applications to new classes of compounds that are not amendable to GC/MS. For compounds of low molecular weights, with good thermal stability and adequate volatility, GC/MS is still a technique of choice. However, for compounds with critical thermal and chemical stability and low volatility the emergence of LC/MS has allowed both qualitative and quantitative analysis that no one had dreamed possible only a decade ago. This chapter is not intended to be a comprehensive compilation of reported applications in the pharmaceutical industry. Rather, it concentrates on several selected examples to provide a useful description of the capabilities and limitations of the technique. Applications to problems such as complex mixture analysis and general structural identification and characterization are covered. A listing of key LC/MS applications can be found in Table 2.

II. LC/MS INTERFACES

A. Overview

1. Why an Interface Is Needed

LC/MS is an integrated hybrid analytical system rather than a hyphenated device. The LC plays a role in preparation, separation, and introduction of sample components to the mass spectrometer; the mass spectrometer is a detector for the LC, garnering both qualitative and

TABLE 2 Applications of LC/MS in the Pharmaceutical Industry

Developmental stages	Information needed
Discovery of drug compound	(a) fast LC/MS for monitoring of target compounds (b) high-resolution mass spectrometry for structural proof and characterization of lead compounds and drug candidates (c) *in vitro* pharmacokinetic and metabolic investigations
Chemical development and scale-up synthesis of drug compound	(d) structural verification of starting materials, intermediates and final product (e) monitoring of impurity profiles from batch to batch (f) characterization and qualification of drug compound/synthetic impurities as reference standards
Stability testing of drug compound; Stability testing of pharmaceutical formulations and packagings	(g) monitoring of changes in purity and impurity profiles over the stability shelf life formulations and packagings under recommended storage conditions (h) investigation of compatibility between drug compound, formulation excipients, and packaging materials (i) understanding of intrinsic stability and degradation pathways of drug compounds and drug products
Development of stability-indicating analytical methods	(j) testing of chromatographic peak purity (k) establishing of impurity profiles under stressed/accelerated conditions
Drug metabolism studies	(l) identification of drug metabolites under physiological conditions
Pharmacokinetic studies	(m) quantitation of drug compounds and metabolites in biological matrix
Toxicological studies	(n) monitoring of impurity profiles throughout the course of study
Marketed product support	(o) continuous monitoring of impurity profiles on drug products and drug substances under long term storage conditions (p) investigation of consumer complaint samples such as drug discoloration issue (q) investigation of counterfeit drug products

quantitative information. The use of a mass detector for peak detection in a chromatographic process offers some special advantages. The most important one is that it can provide a wealth of structural information on the analyte. This information is much more specific than that which can be obtained using a photodiode array detector. For LC, there are a number of widely used detectors such as the ultraviolet-visible spectrophotometer, fluorimeter, refractive index, electrochemical and radioactivity detectors. Each detector has its own niche and offers a range of applications based on its sensitivity, selectivity, and specificity. The successful development of various interface technologies for coupling LC with MS has made this method of detection indispensable in many industries.

Figure 2 shows a chronological display of significant events in the history of MS. However, to define the exact origin of LC/MS is rather difficult. The recognition of the potential of on-line coupling of LC with MS dates back to the 1960s.[5] Interfacing LC with MS is inherently much more difficult than the coupling of GC with MS. The most challenging technical problems arise from the introduction of liquids into a high vacuum system. The mass flow in an LC is tens of thousands times higher than that in a GC. For appropriate performance, typical operating pressures in the mass analyzer region of a mass spectrometer should be in the range of 10^{-8} to 10^{-10} atm (10^{-3} to 10^{-5} Pa). Otherwise, ions will collide with neutral molecules or atoms and be pumped away before reaching the detector. The precipitous drop in pressure at the end of an LC column to

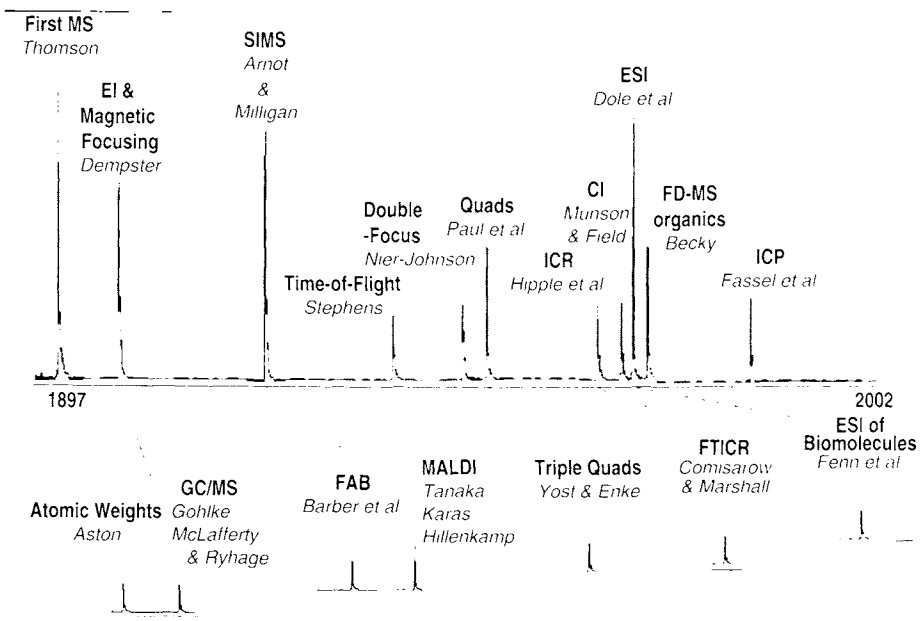


FIGURE 2 MS history (courtesy of http://masspec.scripps.edu/information/history/index.html).

the mass analyzer inside a mass spectrometer would cause enormous problems for the vacuum system if there were no interface.

For example, if the mobile phase is water and a narrow-bore column is used with a flow rate of 0.2 mL/min, it would correspond to 11.2 mmol/min of water, or an equivalent flow rate of 0.272 L/min of gas at ambient condition. This volume of water would expand to 2.72×10^7 L/min of gas at 10^{-8} atm. Such a flow is exceedingly high and cannot be accommodated by even the most sophisticated pumping systems (the theoretical pumping capacity of a cryogenic pumping system is 10^7 L/min). Furthermore, in this example, the tremendous expansion of the HPLC solvent would dilute the analytes to such an extent that even if the pumping system could handle the expanding solvent vapor, the sensitivity would be reduced catastrophically and render the technique useless for many practical applications. An LC/MS interface needed to be invented to remove most of the liquid eluting from the column, and at the same time to prevent dilution and loss of analytes.

2. Characteristics of LC/MS Interfaces

Table 3 shows the objectives and requirements for an ideal interface from the perspectives of both the mass spectrometer and the liquid chromatograph. An ideal LC/MS interface should have the following characteristics:

(1) Allow efficient and precise sample transfer from the LC into the MS with little destruction or loss of analytes.
(2) Permit a wide choice of LC methods and MS operating conditions.
(3) Retain chromatographic peak integrity with minimal peak broadening.
(4) Provide speed, convenience and reliability.
(5) Require minimal instrument maintenance and operator training.

Many interfaces have been developed to meet these demanding challenges. Some of these coupling methods, such as the moving belt or the particle beam interface, are based on the concomitant elimination of the solvent before it enters the mass spectrometer. Other methods such as direct liquid introduction (DLI) or continuous flow FAB rely on splitting the flow of the liquid that is introduced into the interface in order to obtain a flow that can be directly infused into the ionization source. However, these types of interfaces can only handle a fraction of the liquid flow from the LC.

A number of interfaces such as thermospray (TSP), ionspray (IS), atmospheric chemical ionization (APCI) and electrospray (ES) can tolerate much higher flow rates without requiring that the flow be split at the end of the LC column. Ions that are produced in atmospheric pressure ionization sources are moved directly into the mass spectrometer through small apertures.

TABLE 3 Objectives and Requirements for the LC/MS Interfaces

Objectives	Requirements
MS as a detector	
(a) Tolerant of high pressures	(a) Minimal restriction to LC conditions
(b) Selectivity	(b) Sufficient resolution for unambiguous peak identification
(c) Sensitivity	(c) High efficiency in the ionization step and the subsequent ion transmission
(d) Structural identification and characterization	(d) Capable of handling chemically and thermally labile compounds; capable of handling low volatility compounds; versatile in performing various MS/MS experiments
(e) Reliability and reproducibility	(e) Consistent in ionization, ion transmission and detection; user friendly software; and good dynamic range
LC as a separation and sample introduction technique	
(f) Selectivity of separation: sufficient separation so that isobaric compounds can be resolved for unambiguous peak identification by mass spectrometer	(f) Minimal restriction to MS conditions: desirable volatile buffer and compatible flow rate
(g) Sample clean-up and concentration device, eliminate solvent front and salts which cause ion suppression	(g) Retain chromatographic peak integrity; no band-broadening on the chromato graphic peaks; the response should be proportional to the concentration or amount of analyte

B. Development of LC/MS Interfaces

In this section, a brief overview is presented of the development of various LC/MS interfaces. However, it is not intended to include an exhaustive presentation of the working principles of the interfaces. Some excellent reviews of these techniques can be found in a number of articles published by Niessen and co-workers.[6–8]

1. Moving Belt Coupling

The moving wire interface was developed by Scott et al.,[9] and the moving belt interface by McFadden et al.[10] This was the first commercial interface for LC/MS, introduced in 1977. In both of the techniques, the eluent is deposited onto a stainless-steel wire, or a plate usually made of polyimide (known as Kapton), followed by the removal of the solvent in vacuum. The residual solid analyte is vaporized into an ionization

source. The heating for solvent removal and volatilization of solid analyte is usually achieved by passing an electric current through the wire.

The moving wire device has a number of major shortcomings. Due to the small surface area of the stainless-steel wire, such as available from a 0.1 mm diameter wire, the device can only accommodate about 10 µL/min eluent which results in poor sensitivity. The system is difficult to operate in a continuous mode. Modification of the moving wire approach has led to the invention of a continuous moving belt, which offers improved transfer efficiency and therefore higher sensitivity. The moving belt interface is capable of handling up to 1 mL/min of mobile phase.

One of the advantages of the continuous moving belt interface is its ability to ionize the solid analyte using either electron ionization (EI) or chemical ionization (CI). In addition to EI and CI, direct ionization from the transport surface has been reported using FAB,[3] which normally reveals both relative molecular mass and structurally fragmented ions. Applications using surface ionization techniques such as laser desorption (LD) together with the moving belt interface have also been successfully reported.[11,12]

2. Direct Liquid Introduction (DLI)

The DLI interface became the second commercially available LC/MS interface in 1981. The liquid eluent is introduced into the ion source through a capillary or a pinhole diaphragm.[13,14] As the name implies, the analyte in DLI LC/MS is introduced from solution into the MS ion source.[15] When sufficient energy is given to the solution, the preformed ions in solution such as protonated molecules, deprotonated molecules, cationized molecules and solvated ions can be desorbed into a mass spectrometer while the bulk solvent is vaporized and eliminated by the vacuum system.

Maximum flow-rates compatible with DLI interfaces are in the range of 50 to 100 µL/min. Microbore columns (<1.0 mm i.d. column) operating at 5 to 100 µL/min are ideally suited for DLI LC/MS. A flow splitter is required to couple conventional LC with a DLI interface so that only a fraction of the total eluent is introduced into the mass spectrometer. Splitting the flow outside the mass spectrometer results in loss of sensitivity, an undesirable consequence. Henion and co-workers[16] have reported an approach in which the splitter is incorporated into the desolvation chamber of the mass spectrometer. The removal of solvent is achieved by diverting the vapor generated by the solvent without loss of sample and, therefore, sensitivity. Excess pressure inside the mass spectrometer is therefore avoided while higher flow-rates can be accommodated.

3. Thermospray

TSP is a breakthrough LC/MS interface capable of effectively removing solvent from the sample matrix (mobile phase) through a heated

capillary vaporizer. It readily accommodates reversed-phase LC eluents at conventional flow-rates (0.5–2.0 mL/min). Sensitivity of on-column mass at picogram levels has been reported.[4] The basic TSP interface is comprised of a heated vaporizer, a desolvation chamber, and an ion extraction skimmer. When a sample solution is pumped into a heated stainless-steel capillary, rapid evaporation of solvent from the liquid surface occurs, resulting in an ultrasonic spray of vapor and charged droplets. Disintegration of the charged droplets occurs repetitively due to continuous evaporation of solvent and the Coulombic repulsion between like charges. The process eventually causes ions, as well as neutral molecules, to be released from the surface of the microdroplets. The ions are extracted and accelerated toward the analyzer by an electrostatic system voltage. Therefore, TSP is both an interface and an ion source, which makes a separate ionization source unnecessary.

Ions in the TSP process can also be produced in a two-step manner similar to conventional CI.[17] The reagent ions, e.g., NH_4^+, formed from electrolytes such as ammonium acetate, react with analyte molecules in the gas phase to generate positive analyte ions. This process can also be used with equal facility to generate negative analyte ions.

The TSP interface was very popular and attractive to chromatographers in the 1980s, as a result of its ease of operation and dependable performance. Commercial TSP LC/MS systems are equipped with an electron emitter filament to enhance the CI process.

A modification/enhancement of the TSP is the plasmaspray interface for discharge ionization which has also been commercialized. A TSP system can be operated in three different modes: (a) filament-off mode, i.e., TSP ionization mode, (b) the filament-on mode, and (c) the discharge ionization mode.

4. Continuous-Flow FAB

The principle of FAB, less frequently referred to as liquid secondary ionization mass spectrometry (LSIMS), is very similar to secondary-ion mass spectrometry (SIMS). However, FAB utilizes a liquid matrix, such as glycerol, in which a sample is dissolved. The matrix is used to enhance sensitivity and ion current stability.

Barber et al.[3] introduced FAB in 1981. In this technique, bombardment of a liquid target surface by a beam of fast atoms such as xenon or argon, causes the continuous desorption of ions that are characteristic of the liquid. In a typical FAB spectrum, the analyte ion is usually formed as protonated or cationized ions in positive FAB, and deprotonated ions in negative FAB mode. A few fragmented ions may also be formed. The spectrum usually contains peaks from the matrix, such as protonated matrix clusters of glycerol if it is used as the matrix solvent. FAB utilizes a liquid matrix such as glycerol. The matrix is used to enhance sensitivity and ion current stability.

The most commonly used FAB interface in LC/MS is known as continuous-flow fast-atom bombardment (CF-FAB) ionization, in which the fast atoms or ions are directed at a target along which the LC eluent flows.[18] In a CF-FAB, the LC eluent, mixed with a FAB matrix such as 5% aqueous glycerol, is continuously introduced and deposited on the tip of a FAB probe. The maximum flow rate is in the range of 5 to 15 μL/min. A comprehensive review of the principles and application of CF-FAB for LC/MS has been written by Caprioli.[19]

5. Particle Beam Interface

Particle beam interface is a device capable of separating solvent from solute without losing much of the solute. The interface has been developed based on the original work of Browner and co-workers.[20,21] The eluent from LC is passed through a nebulizer and converted into a spray of fine liquid droplets with high velocity. The nebulization is either assisted pneumatically, or thermally. Solvents begin to evaporate and the solute starts to concentrate. When the liquid droplets exit the heated chamber, they leave as a fast-moving particle beam. The beam entering the ion chamber is subjected to EI or CI ionization.

6. Electrospray Ionization

ESI has become the most commonly used interface for LC/MS. It was recognized by John Fenn and co-workers[22] as an important interface for LC/MS immediately after they developed it as an ionization technique for MS. ESI transforms ions in solution to ions in the gas phase and may be used to analyze any polar molecule that makes a preformed ion in solution. The technique has facilitated the ionization of heat-labile compounds and high-molecular-weight molecules such as proteins and peptides. ESI is a continuous ionization method that is particularly suitable for use as an interface with HPLC. It is the most widely accepted soft-ionization technique for the determination of molecular weights of a wide variety of analytes and, has made a significant impact on drug discovery and development since the late 1980s.

A couple of excellent reviews on the ionization mechanism of ESI can be found in the literature.[23,24] The following major events occur in an ESI process:

(a) Nebulization of sample solution into electrically charged droplets.
(b) Drastic reduction in the volume of the charged droplet by solvent evaporation.
(c) Disintegration of the droplets which results in highly charged micro-droplets capable of ion formation.
(d) Production of gas phase ions from these charged droplets.
(e) The ions formed are extracted, focused, and transported into the mass spectrometer for mass analysis.

A solution of the sample is sprayed through a capillary needle maintained at a high electric potential of approximately 5 kV. The voltage on the needle causes the liquid spray to be charged as it is nebulized. The droplets evaporate in a stream of dry gas and applied heat, in a region maintained at a vacuum of several Torr. As the droplet decreases in size, the charge density on the droplet surface increases. When the Coulombic repulsion between like charges on the surface overcomes the forces of surface tension (the Rayleigh limit[25]), the droplet disintegrates explosively to form second-generation liquid droplets. This process occurs repeatedly so that ions leave the droplet and are directed into the mass analyzer. A schematic presentation of the ESI process is illustrated in Figure 3. The ESI process bears some similarity to other LC/MS interfaces such as TSP[26,27] and ion evaporation.[28]

The most important feature of an ESI spectrum is that multiply charged ions can be formed. As a result, mass analysis of large molecules becomes feasible. For example, under electrospray positive ion monitoring, apo-myoglobin, which has a molecular weight of 16 951.5 atomic mass unit (amu), usually produces a series of ions with charge states from +8 to +27, and mass peaks from about 600 to 2000 amu. Figure 4 shows the ESI positive ion mass spectra for angiotensin I corresponding to charge states from 1, 2, 3, and 4. The charge state can be recognized by the difference in the *m/z* values between two adjacent isotopic peaks. A difference in the *m/z* values of 1 indicates a charge state of 1; a difference of 0.5 corresponds to a charge state of 2; and so on.

Typical mobile phases compatible with electrospray are methanol, acetonitrile, isopropanol, and volatile buffers. Many other solvents can also be used in ESI, including $CHCl_3$ and THF. Although 100% water can be used, better sensitivity is obtained with some organic modifier

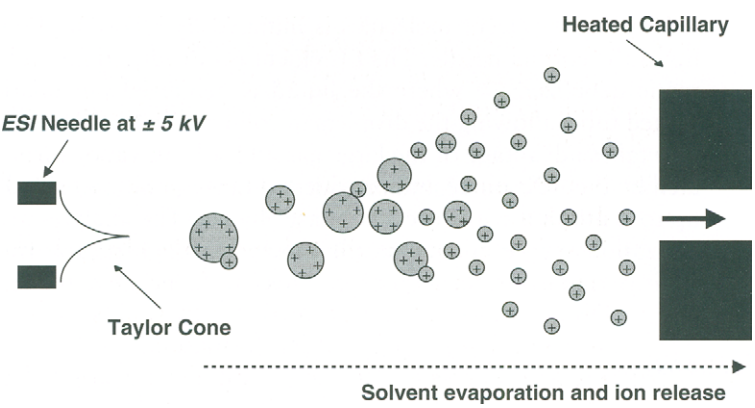


FIGURE 3 Schematic presentation of ESI process.

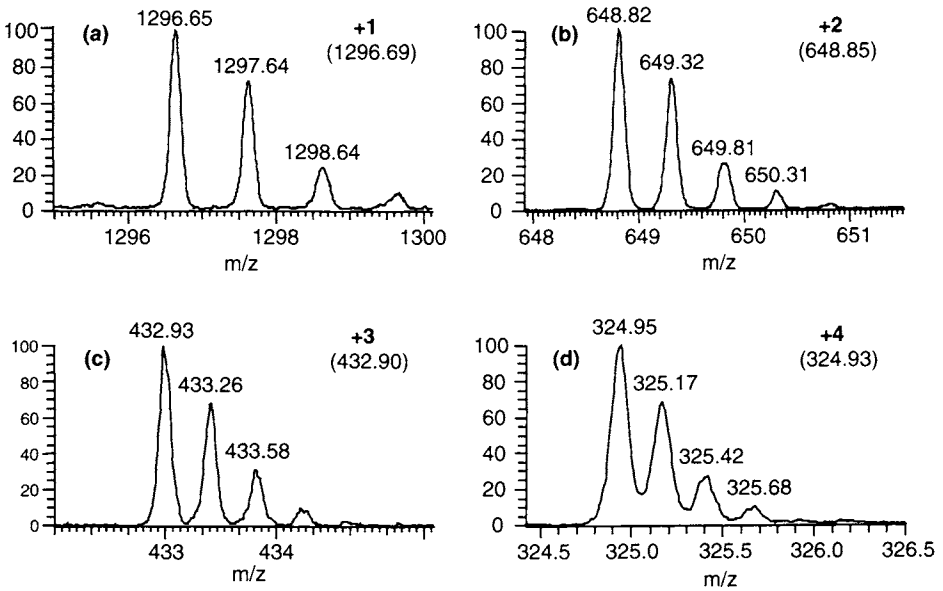


FIGURE 4 **ESI positive ion mass spectra of angiotensin I with charge states of +1, +2, +3, and +4 (courtesy of Thermo Finnigan).**

being present. Even 5–10% of MeOH or ACN significantly increases the stability of the nebulization process.

7. Atmospheric Pressure Chemical Ionization

In contrast to the ESI process where ions are primarily formed by desorbing preformed ions from a solution, APCI is an ionization technique in which ions are produced at atmospheric pressure, by gas-phase ion-molecule reactions between analyte molecule and solvent-based reagent gas. A graphical illustration of the APCI process is displayed in Figure 5. Ionization of solvent molecules is initiated by a corona discharge at the tip of the corona needle. The LC eluent is introduced into a heated pneumatic nebulizer,[29,30] where the liquid is nebulized pneumatically into a heated tube, allowing the droplets to collide with the hot walls. The interface takes advantage of the large gas and solvent vapor throughput tolerated by the API source and provides routine operation using LC flow rate up to 2 mL/min with reversed-phase eluents. The analytes arriving in the ionization source are chemically ionized through proton transfer in the positive mode and through proton loss in the negative mode.

C. Ionizations

As discussed above, ionization may occur in the LC/MS interface or in a separate ion source. During the development of commercial LC/MS

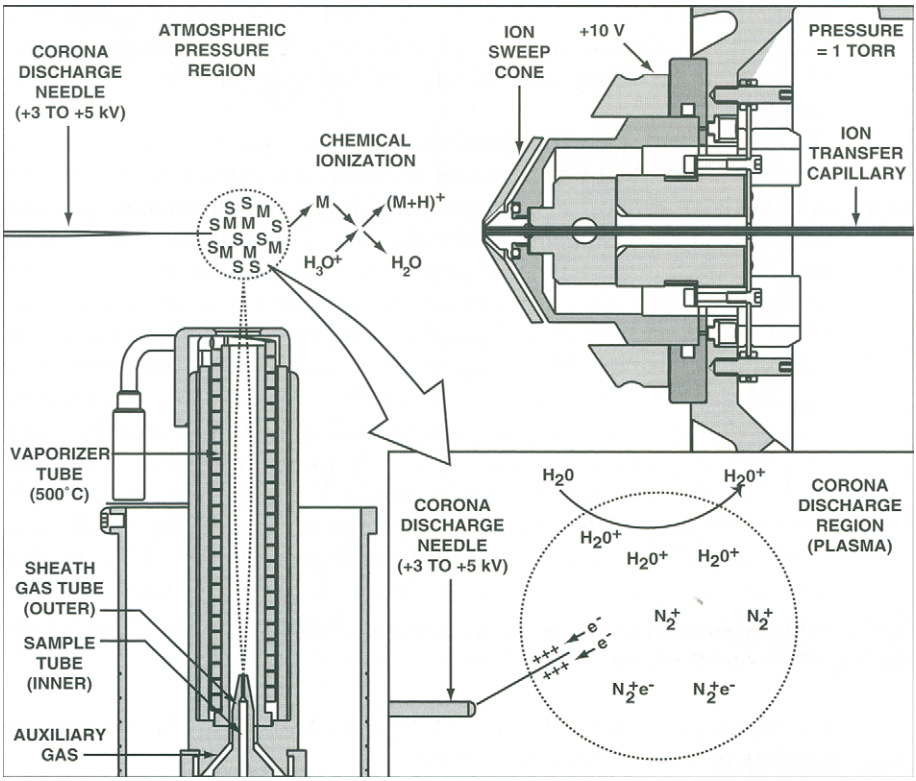


FIGURE 5 Schematic presentation of APCI process (courtesy of Thermo Finnigan).

interfaces, much emphasis was placed on the designs of interface between LC and MS. For example, the moving belt and particle beam systems are interfaces between the LC and EI and/or CI sources. DLI is an interface in which the ionization is achieved by solvent-mediated CI. The thermospray nebulizer is the interface for electron beam- or electric discharge-induced CI. The heated pneumatic nebulizer is the interface of choice for LC/MS with APCI.

EI produces predominantly +1 charge ions by ejecting an electron off the gaseous, neutral molecules. For compounds with high electron affinity, capture of an electron by the neutral analyte molecules to produce −1 charge ionic species may also be observed in the EI process. The analyte molecules must be transferred into the gas phase from condensed phases (solid or solution) before interacting with the electron beam in the ionization region. Heat used for this phase transformation can lead to thermal decomposition of analyte molecules prior to vaporization. Although EI was the primary ionization source for mass spectrometric analysis until the 1980s, its limitation for thermally labile

molecules and large biomolecules left much to be desired and inspired many MS pioneers to develop alternative ionization techniques known today as electrospray and matrix-assisted laser desorption and ionization.

ESI desorbs the preformed ions from the solution phase into the gaseous phase. Many compounds in solution establish an equilibrium with their ionic species. Common ionic forms may include protonated, cationized, or deprotonated molecules. When ES is operated in the positive ion mode, a partial separation of positive and negative ions of the analyte and other electrolytes present in the solution occurs near the capillary tip. The electrosprayed droplets are found to contain excess positive ions. Under the effect of drying gas and heating, the continuous evaporation of solvent molecules occurs, which results in increasing charge density that makes the droplets electrohydrodynamically unstable. The droplets disintegrate when electronic repulsion reaches the Rayleigh stability limit. Further evaporation of solvent from the resulting smaller droplets leads to repeated droplet disintegration, which eventually results in the generation of ions in the gas phase. The ions are often solvated. The solvated ions can be preserved under soft ionization conditions such as electrospray ionization. Therefore in an ESI mass spectrum solvent-adduct ions are frequently detected.

III. MASS ANALYZERS

A. Characteristics of a Mass Analyzer

A mass analyzer is the device that resolves different gas-phase ions according to their mass-to-charge ratios. There are many different mass analyzers, and most of them can be easily coupled to an HPLC system and function as HPLC detectors. The most commonly used mass analyzers include Sectors (magnetic and electrostatic), Quadrupoles (single or triple quadrupole), Quadrupole Ion Trap, Fourier Transform Ion Cyclotron Resonance and Time-of-Flight. The separation of ions can be achieved either in a temporal or spatial dimension. Some mass analyzers allow the simultaneous detection of all ions, such as the Fourier transform ion cyclotron resonance mass analyzer. Other mass spectrometers, such as the quadrupole mass analyzers, act as mass filters so that only ions with particular mass-to-charge ratio can register a signal at any particular time. The time-of-flight (TOF) mass analyzer requires the ions to be produced simultaneously and all ions to be accelerated to the same kinetic energy before they can be separated in the flight tube. However since ionization in a typical LC/MS operation generates a continuous stream of ions, the ions are generally introduced into the TOF mass analyzer orthogonally before they are pulsed out of the source region.

The type of mass analyzer largely determines the information obtained from a given experiment, and to what extent that information can be translated into knowledge. The performance of a mass analyzer is evaluated by its capability to resolve neighboring ions, mass accuracy, upper mass limit, and transmission efficiency. A summary comparing the advantages and disadvantages of different mass analyzers is given in Table 4.

Instruments combining several analyzers in sequential order are very common. This combination allows mass spectrometry and mass spectrometry experiments (MS/MS) to be carried out. Modern MS/MS includes many different experiments designed to generate substructural information or to quantitate compounds at trace levels. A triple quadrupole mass spectrometer allows one to obtain a daughter ion mass spectrum resulting from the decomposition of a parent ion selected in the first quadrupole. The MS/MS experiments using an FTICR or ion trap, however, are carried out in a time-resolved manner rather than by spatial resolution.

B. Working Principles of Mass Analyzers

A brief description of the working principles of commonly used mass analyzers is given below. For a more comprehensive discussion of the principles of these mass analyzers, excellent reviews can be found in the literature.[31,32]

1. Sector Mass Analyzers

In sector instruments, magnetic sectors are generally coupled with electric sectors to correct the difference in the initial energetic and spatial distribution for the incoming ions. Magnetic sector mass analyzers are fundamentally momentum analyzers. Ions in a magnetic field traverse in a circular path in a plane perpendicular to the direction of the magnetic field. Ions are dispersed spatially according to their momentum-to-charge ratio. Ions of different mass but of the same kinetic energy follow different trajectories in a fixed magnetic field. The strength of the magnetic field, if varied, will allow ions with different masses to follow the trajectory that will lead them eventually to the detector. Therefore only ions with a given mass-to-charge ratio will be able to reach the detector in any given magnetic field.

2. Quadrupole Mass Analyzers

The quadrupole is a device which takes advantage of the stability of the ion trajectories to separate ions in accordance with their mass-to-charge ratio. Quadrupole analyzers consist of four rods with circular or, ideally, hyperbolic surface sections. The four rods are arranged

TABLE 4 Pros and Cons of Mass Analyzers

Mass analyzers	Advantages	Disadvantages
Sector	• High resolution and accurate mass measurement, generation of elemental compositions • High-energy CID MS/MS for generation of sub-structure information • Mass range up to 20 000	• The system is expensive and often complex • Low sensitivity • Coupling to LC is technically demanding • Slow scan speed
Quadrupole	• Energy and spatial distribution of ions produced in the ion source is not critical • Low cost and easy to couple to LC • Tandem MS experiments available in triple quadrupole or Q-TOF systems for sub-structure information and/or quantitative analysis • Vacuum system demands are minimum	• Low resolution and low accuracy in mass measurement except in Q-TOF systems • Mass range limited to approximately 4000
Ion-trap	• Energy and spatial distribution of ions produced in the ion source is not critical • Inherent tandem MS capabilities for generation of sub-structure information • Low cost and easy to couple to LC • Vacuum demand is minimum	• Low resolution and low accuracy in mass measurement
FTICR	• Ultra high resolution and high mass accuracy, elemental composition can be obtained • Inherent tandem MS capabilities for generation of sub-structure information • Mass analysis is nondestructive • High sensitivity • Mass range > 20 000 • Fast scan speed	• High purchase price and high cost in maintenance • Vacuum requirements are demanding and coupling to LC is difficult • Fast computing required
TOF	• High resolution and mass accuracy when operated in reflectron mode or in Q-TOF systems, elemental composition can be obtained • Tandem MS available for generation of sub-structure information or quantitative analysis • High sensitivity • Very fast scan speed • Unlimited mass range	• Initial energy and spatial distribution must be corrected for ions • High-performance electronics needed

precisely parallel, and equally spaced, around a central axis, i.e., in a square array. The ion beam is focused down the axis of the array and an electrical potential of fixed (DC) and radio frequency (RF) components is applied to the diagonally opposed rods. For a given combination of DC and RF components, ions of one specific *m/z* ratio have a stable path down the axis. All others are deflected to the sides and lost before reaching the mass detector—ions with too low a value of *m/z* spiral out of control and crash into the positively charged rods, while ions with too high a value of *m/z* spiral out of control and crash into the negatively charged rods. Mass scanning spectrum is generated by changing the DC and RF components of the voltages while maintaining a constant ratio.

3. TOF Analyzers

Ion separation in a TOF mass analyzer is based on Newton's third law. The underlying principle is that ions of different masses with equal kinetic energy have different velocities. If there is a fixed distance for the ions to travel, the time of travel is proportional to the square root of the mass-to-charge ratio of the ions. To measure the time of flight, ions are introduced into the mass spectrometer in discrete packets so that a starting point for the timing process can be established. Ion packets are generated either through a pulsed ionization process or through a gating system in which ions are produced continuously, but are introduced only at given times into the flight tube.

4. Ion-Trap Analyzers

The analyzers discussed above are all mass filter analyzers, with spatially separated input and output. There is another class of mass spectrometers in which mass analyzers store the ions for subsequent mass analysis. The most common of these is the quadrupole ion-trap, which is effectively a quadrupole mass filter bent around on itself. This quadrupole-type device is composed of a ring electrode placed between two end-cap electrodes. The end caps are either held at ground potential or have an RF voltage applied to them, while an RF voltage is placed on the ring electrode. As a result of this specific geometric arrangement, the hyperbolic surfaces of the three elements form a three-dimensional quadrupole analyzer. Holes in the end caps allow ion injection into the device, and ion ejection out of the device to the detector. In the ionization step, the RF voltage on the ring electrode is set low enough so that the ions within the mass range of interest are trapped within the device. The RF voltage on the ring electrode is raised following ionization, and the ions of successively higher masses are ejected from the ion trap into an electron multiplier detector. A particular advantage of ion-trap MS is the so-called MS^n technique; in such a process, a fragmented ion from its precursor is selected and subjected to collision induced dissociation (CID) so

that structural information on the precursor ion can be obtained. Low mass cut off is a problem in MS^n, but may be overcome by using additional isolation and excitation steps.

5. Ion Cyclotron Mass Analyzers

Fourier transform ion cyclotron mass analyzers (FTMS) are based on the ion cyclotron resonance (ICR) principle. Ions are stored and analyzed inside an ICR analyzer cell that is located in a homogeneous region of a superconducting magnet. Under the influence of a magnetic field, ions experience a force perpendicular to their motion that makes them move in a helical direction. The time required for ions to complete one helical movement is proportional to the mass-to-charge ratio of the ions. Faster ions move in larger helices (or orbits), and slower ions in smaller helices. Therefore, the cyclotron frequency for ions with the same mass-to-charge ratio is always the same. A frequency analysis coupled with a Fourier transform of the signal generates information for the mass-to-charge ratio of the ions. The advantages of an FTMS instrument are its remarkably high resolution and the ability to measure molecular mass accurately, from which the elemental composition of an unknown compound can be derived.

IV. PRACTICAL CONSIDERATIONS IN SELECTION OF LC/MS PARAMETERS

The most commonly used LC/MS interfaces in pharmaceutical analysis are ESI and APCI. An ESI interface on the majority of commercial mass spectrometers utilizes both heat and nebulization to achieve conditions in favor of solvent evaporation over analyte decomposition. While ionization in APCI occurs in the gas phase, ionization using ESI occurs in solution. Attributes of a mobile phase such as surface tension, conductivity, viscosity, dielectric constant, flow rate and pH, all determine the ionization efficiency.[33,34] They therefore need to be taken into consideration and controlled.

A. Flow Rate

It is well known that UV detectors used in liquid chromatographs are concentration-sensitive devices. Injection of the same mass of a particular compound onto two columns with identical plate number and length but different inner diameters, will result in a higher response from the column with the smaller inner diameter. The gain in the signal is inversely proportional to the square of the ratio of the inner diameters of the two columns. The situation is different for a mass spectrometer, which is a mass-flow sensitive detector. Under constant flow conditions,

the mass spectrometric response is proportional to the concentration of analyte ions reaching the mass detector. If the analyte ions are continuously introduced into a mass spectrometer, the signal will decrease as the flow rate decreases.

Niessen[31] recommended that efforts to improve concentration detection limits should be directed at improving the mass-flow to the mass spectrometer. Hopfgartner et al.[35] also confirmed that there was no gain in concentration detection limit by using microbore columns vs. a conventional column with a flow split as the gain in the analyte concentration obtained by using a smaller diameter column comes at the expense of a reduced flow-rate entering the electrospray interface. Another factor arises from the requirement that similar reduction of the injection volume must be adjusted with the reduced column diameter. The two factors work concurrently but antagonistically, leading to reduced mass reaching the mass detector.

ESI, however, has shown that ionization and sampling efficiency is inversely proportional to the flow rate.[36] Observations of reverse mass-flow dependence behavior for the ESI interface have been reported.[37,38] The explanation of these disparate observations can be attributed to an improvement in the ionization and sampling efficiency achieved in the ESI process at lower flow rates. When the gain due to higher ionization and ion sampling efficiencies exceeds the loss of sensitivity due to mass-dependence from mass detector, an improvement in the overall response results. An important event in electrospray mass spectrometry is the sampling of analyte ions formed in the atmospheric-pressure ionization chamber. Although modern ESI interfaces can handle flow rates up to 1 mL/min without flow splitting prior to the interface, the sampling of analyte ions through either a heated capillary or an orifice is actually a split process, which allows only a fraction of the formed ions to eventually reach the mass detector. According to literature research for the period of 1992–1997 reported by Abian et al.,[38] narrow-bore columns were more often used for LC/MS applications. Narrow-bore columns are those with 1 to 2 mm in internal diameters. The popularity of narrow-bore columns can be attributed to their compatibility with common HPLC pumping systems and pneumatically-assisted ESI source requirements. Compared to conventional columns, typical flow rates used in narrow-bore columns are reduced by as much as 90%. Flow rates in the range of 0.2 to 0.4 mL/min are commonly used for ESI LC/MS applications.

Utilization of capillary columns in conjunction with micro ESI devices is becoming a new trend in the field of LC/MS. Capillary HPLC has become a particularly important technique in situations where the supply of analyte is limited, such as in proteomic analysis. According to studies conducted by Smith et al.,[39] only one in a hundred thousand of analyte molecules present in solution eventually reach mass detection in a conventional ESI interface. Smith et al. attributed this poor electrospray

performance to less efficient sampling process. The efficiency can be improved to one of every 390 analyte molecules being detected using nano-ESI tip which is placed 1–2 μm from the MS entrance. However, the use of capillary columns in combination with nano-ESI in pharmaceutical analysis is not required because abundant sample is usually available. Since capillary column/nanoESI requires special configuration of the instrument, it has not yet been accepted as a routine analytical technique for pharmaceutical analysis.

B. Organic Solvent in the Mobile Phase

Mobile phases used in LC/MS for pharmaceutical analysis mostly consist of methanol and/or acetonitrile, and a volatile buffer such as ammonium formate. The viscosity of solvent mixtures varies when the composition ratio changes. It is well known that less viscous solutions lead to more efficient evaporation and ion desorption. Since organic solvents such as methanol and acetonitrile have lower viscosity and higher vapor pressure than water, these solvents are more easily removed in the ESI interface and thus enhance ESI performance. Methanol has also been reported to generate stronger signals than acetonitrile[40,41] in positive ion mode MS. The need to maintain low concentrations of buffer can be understood from a number of publications by Kebarle and Tang.[42–44] They monitored the response of analyte ions as a function of the background electrolyte content in the solution. They reported that the formation of a particular analyte ion can be significantly suppressed by the presence of a second analyte. For example, the intensity of analyte ions such as Bu_4N^+ and CodeineH$^+$ decreased by more than a factor of 10 when the concentration of ammonium acetate was increased from 0.05 to 1 mM. Other references found in the literature[45] discuss the relationship of mobile phase additives such as formic acid, ammonium formate content and the response of an analyte. The optimal response was reported when the additive concentration was between 0.1 and 1 mM. The presence of formic acid enhances the formation of protonated analyte ion, while the introduction of a metal alkali such as sodium acetate can lead to an abundance of sodiated analyte ions. Nevertheless, the concentration of volatile buffers such as formate in the mobile phase is recommended at 10 to 30 mM for practical reasons of having sufficient buffering capacity.

Straub and Voyksner[46] developed a schematic approach for ionization optimization at a variety of pH values and organic modifiers. As a general rule, for every 5–10% increase of organic modifiers in the mobile phase, the mass response will double. A compromise approach must be adopted in choosing the type and ratio of organic modifier so that desirable separation and ionization can be achieved simultaneously. As a general rule, in either methanol- or acetonitrile-based mobile phases, a concentration range of 20% to 80% of either methanol or acetonitrile is optimal.

C. Additives and Buffers

It is well known that nonvolatile HPLC additives should be avoided. These include alkali metal phosphates, borates, citrates, hydrochloric acid, sulfuric acid, alkali metal bases, etc. The influence of pH on the ionization is obvious in the ESI process. Analyte molecules must enter the ESI interface under conditions favorable to the formation of the ions in the solution phase. The general rule is that basic compounds are chromatographed in acidic mobile phases to produce protonated analyte ions, and that acidic compounds are chromatographed in basic mobile phase to generate deprotonated analyte ions. However, the pK_a value of a compound when in the gas phase may be drastically different from its pK_a in solution. The phenomenon of "wrong-way-round" in electrospray ionization[47] may be partially due to this shift in pK_a values during phase transformation. The fact that amino acids can be protonated as well as de-protonated during ESI process in the pH range between 3 and 11 gives an analyst a broad choice of pH values of mobile phase should separation become a more critical issue than sensitivity. Under such circumstances the pH value can be optimized to achieve optimal separation without severely compromising MS sensitivity.

Post-column introduction of a sheath liquid gives more flexibility to the selection of pH values for a mobile phase. Chiron et al.[48] applied post-column addition of tripropylamine to reduce the acidicity of the mobile phase in their study of bentazone and chlorophenoxyacetic acids to enhance ESI negative ion signals. Apffel et al.[49] on the other hand, introduced propionic acid/isopropanol to displace TFA in the mobile phase, and improved the ESI positive ion signals.

Using seven nucleoside antiviral compounds, Kamel et al.[50,51] have reported that ES sensitivity for all seven compounds was increased by a factor of at least 2 when the mobile phase was modified with 1% acetic acid (pH 3.1) rather than 0.1% trifluoroacetic acid (pH 2.3). At constant pH, intensity of the protonated ion increased with increasing pK_a of the analyte. In the positive ionization mode, the sensitivity of these nucleoside compounds as $[M+H]^+$, did not depend on the pH of the solution. In the negative ion mode however, increased sensitivity in $[M-H]^-$ ion was indeed observed with increasing pH values. Temesi and Law[52] also reported a study of the effect of LC eluent composition on mass spectrometric response using electrospray ionization. The study was carried out using 35 compounds with diverse structures. They included strong and weak acids and bases, amphoterics, and polar neutral compounds. The authors concluded that formic acid provided the best all-round responses for all 35 compounds tested, regardless of what organic modifiers were used. Trifluoroacetic acid resulted in reduced responses in the positive ionization mode, and a total absence of signals was the result in the negative ionization mode with trifluoroacetic acid in the mobile phase.

Figure 6 shows the signal response in ESI of Leucine Enkephalin, a pentapeptide, as a function of solvent and additive variation. Although the peptide can be ionized in basic media, acidic pH is much more favorable. This result also confirms that formic acid and acetic acid promote ESI signals far more efficiently than TFA.

D. MS Operating Conditions

Although modern mass spectrometer has evolved to a point that a fully automated system can be set up in an open-access laboratory, some general aspects in their operation should be mentioned. Since large amounts of nitrogen gas are consumed as nebulizing and drying gas, most pharmaceutical companies provide it through internal plumbing systems. Typically, these systems deliver nitrogen gas from a remote nitrogen generator. It is worthwhile to install an in-line filtration device so that moisture and grease can be removed before they reach the mass spectrometer ion source. The highest research-grade of argon gas should be used as the CID gas to avoid possible contamination. For systems in which the spray needle is adjustable, one must examine the influence of needle position on the ionization response. The API probes used in Thermo Finnigan LCQ systems are shown in Figure 7. The *xy* position of the spray needle can be adjusted by using the screw on the ESI flange.

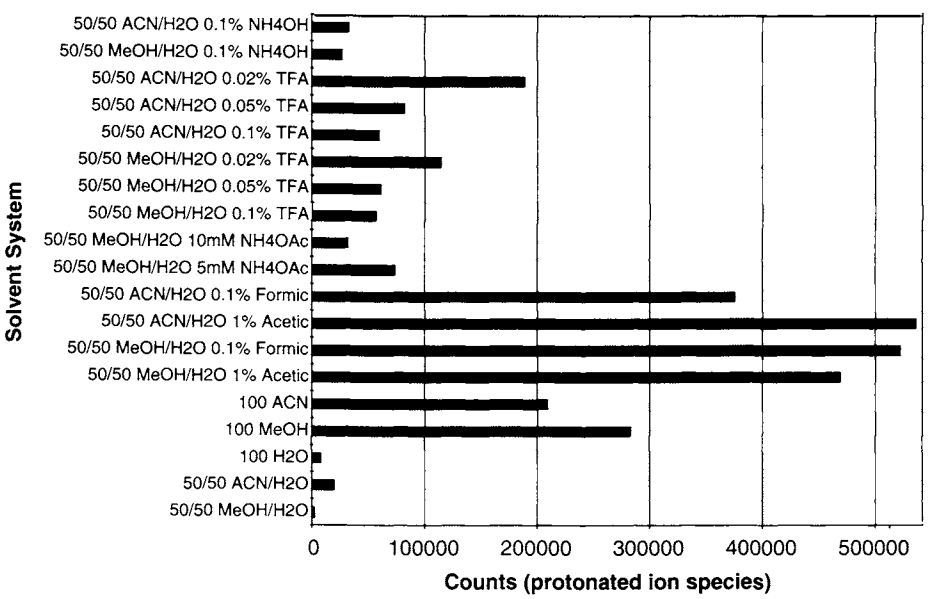


FIGURE 6 **Effects of solvents and additives on ESI response of Tyr–Gly–Gly–Phe–Leu (Leucine Enkephalin) (courtesy of Thermo Finnigan).**

The scan range can have a great impact on the quality of the mass spectrum. Ionization techniques for LC/MS such as particle beam, thermospray, and electrospray inherently generate high levels of chemical noise in their total ion chromatograms and in their mass spectra. Although background subtraction is effective in eliminating most of the chemical noise, it is wise to limit the scan range thereby enhancing the quality of the spectrum. If the molecular mass of the drug is designated as M_r, then the upper m/z limit is recommended as $2M_r + 50$ since dimerization of drug molecules may be one of the degradation pathways. The low end of the m/z limit should be set at a point so that all of the synthetic intermediates and starting materials can be detected. If the method is intended to screen for leachables from packaging materials, the m/z range should also be set accordingly. A number of trial or scouting LC/MS runs can be made to obtain preliminary information so that more optimal parameters can be set up for subsequent experiments.

E. Sample Solution Effects

In principle, enhanced sensitivity can be achieved when the on-column focusing of the analyte is possible. The sample is preferably dissolved in a solvent with lower eluotropic strength compared to that of the mobile phase at the start of a chromatographic run. A solvent with

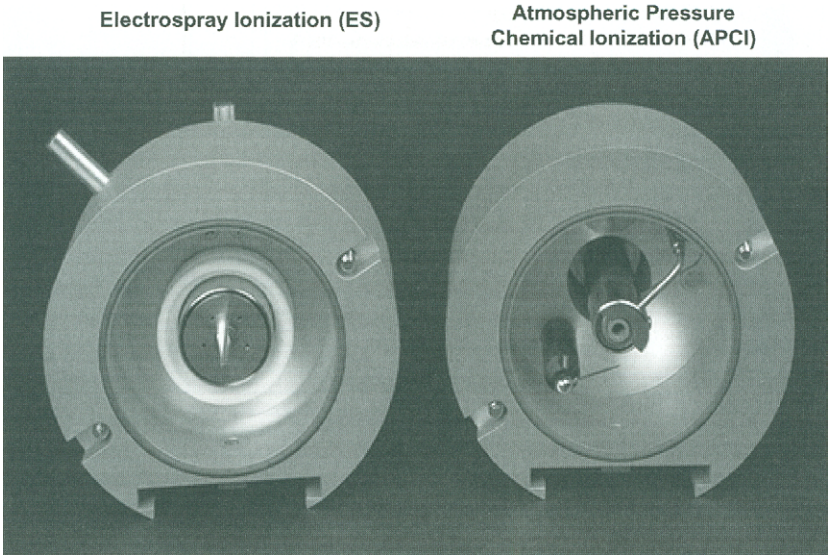


FIGURE 7 API probes used in Thermo Finnigan LCQ systems (courtesy of Thermo Finnigan).

much higher eluotropic strength, will likely cause chromatographic anomalies such as peak distortion, splitting or broadening. Another factor that may cause peak distortion or splitting is a mismatch of the pH of the sample solution and that of the mobile phase.

The majority of components in a pharmaceutical formulation are excipients. Some of them, when extracted, may have a deleterious effect on the mass analysis process. For example, polyethylene glycol, a water-soluble polymer, can span several minutes of the total ion chromatogram. The presence of PEG ions makes identification of trace level impurities extremely difficult. Figures 8(a) and (b) compare the UV and TIC traces. Three unknown peaks in between retention times of 8 and 12 min were observed in the LC/UV chromatogram. Unfortunately, in the same time region, the total ion chromatogram is dominated by the signals from polyethylene glycol, making detection and the assignment of signals to the unknown peaks an arduous task. Figures 9(b)–(d) represent the extracted mass spectra from the retention time regions corresponding to these three unknown peaks. Five strong signals in Figure 9(b) have been observed. Their *m/z* values are 236, 459, 476, 503, and 520. The ion at *m/z* of 236 is "chemical background noise"; the ions at *m/z* of 459 and 476, are the protonated and ammoniated ions of an oligomer of polyethylene glycol; the ions at *m/z* of 503 and 520 are another pair of

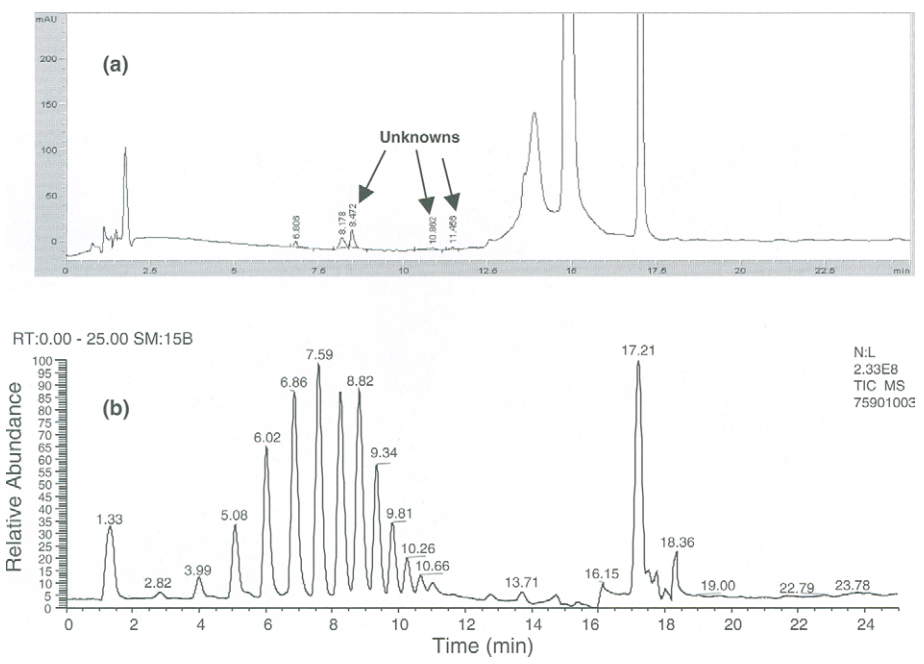


FIGURE 8 PEG effect: (a) LC/UV chromatogram and (b) total ion chromatogram.

signals corresponding to another oligomer of polyethylene glycol. The oligomers of two adjacent polyethylene glycol peaks are characterized by an increment of 44 amu. In addition, each oligomer shows a pair of ions, one is the protonated molecular ion, while the other is the ammoniate molecular ion. The signals for all of the polyethylene glycol oligomers have been assigned and labeled in Figure 9(a). There is a weak signal in Figure 9(b) with an *m/z* value of 302. This signal does not fit the pattern for polyethylene glycol oligomers. The *m/z* value of this ion is 40 amu lower than that of the API, suggesting that it is a degradation product formed through a dealkylation reaction leading to the loss of an allyl group from the drug compound.

Other drug excipients, such as plasticizers, may also have an adverse impact on LC/MS performance and interpretation. Plasticizers such as triethyl citrate, *tert*-butyl phthalate, and some others, can act as bases to compete for protons during the ion generation process. Selecting the optimal solvent or designing sample work-up strategies to discriminatorily dissolve components of interest is a very important step towards the generation of high quality mass spectrometric data. A more detailed discussion regarding sample preparation for pharmaceutical dosage forms is presented in chapter 5.

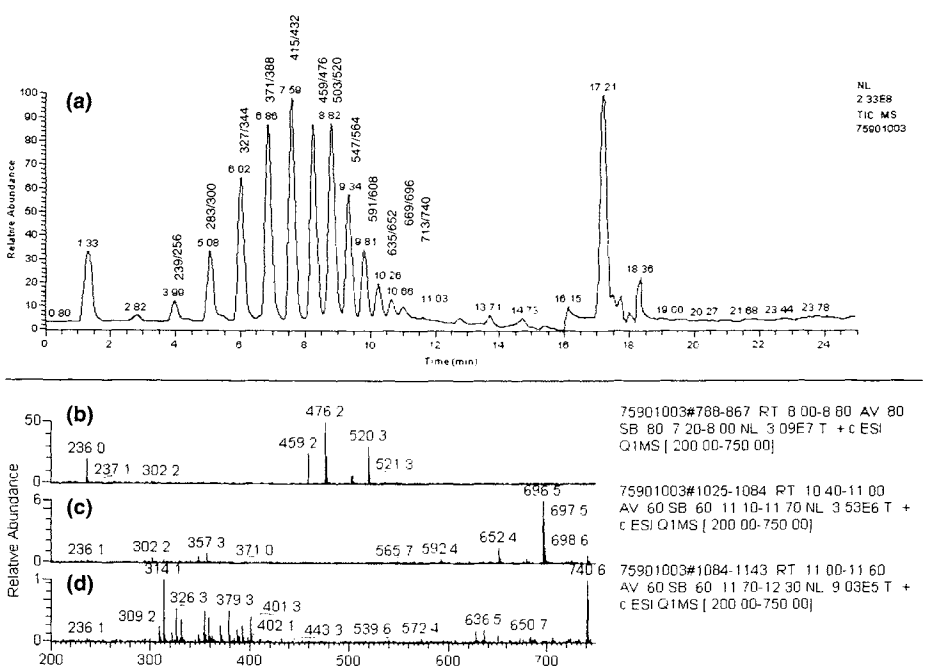


FIGURE 9 **Extracted mass spectra of peaks of interest in the presence of PEG signals.**

V. PEAK TRACKING BETWEEN LC/UV AND LC/MS METHODS

Preferably, identification of impurities in drug substances and drug formulations by LC/MS is carried out using the same chromatographic method used to detect the unknown initially. However, nonvolatile buffers and ion-pairing reagents are commonly used to mediate chromatographic separation and improve its performance such as high sensitivity in the HPLC methods designed for assay and the determination of impurities. Since nonvolatile buffers are not compatible with LC/MS interfaces, peak tracking between different chromatographic methods has become one of many challenges one must deal with.

A. Developing an Equivalent HPLC Method

A practical approach to peak tracking is to develop an LC method for LC/MS application which has the same or similar peak elution order as the original method. Phosphate buffers can be replaced by ammonium formate (pH 2.8–4.8), ammonium acetate (pH 3.8–5.8), or ammonium bicarbonate (pH 8.2–10.2). Figure 10(a) shows a chromatogram for a neutraceutical mixture using a phosphate-buffered mobile phase. When phosphate is replaced with formate at the same pH, chromatographic selectivity is often preserved. Identical elution order, and even the same retention times, can be seen when comparing Figure 10(b) with (a). Ion-pairing agents such as sodium dodecyl sulfate (SDS) can be substituted with one that is MS friendly. There are a number of ion-pairing reagents, which at appropriate concentration levels, can maximize HPLC separation without compromising ESI/MS sensitivity. Trifluoroacetic acid has been known since the early 1980s for its characteristics in achieving better separation for peptides in reversed-phase HPLC process. In the last few years heptafluorobutyric acid and pentafluorobutyric acid have been used to improve HPLC separation at concentration levels such as 0.05% for small molecules and 0.005% for peptides.

Once an appropriate, MS-friendly, LC method is developed, information such as the UV spectrum and percent peak area can be used to establish peak correlation between LC/UV and LC/MS methods.

B. "On-line" Peak Trapping and Elution

A well-known method for peak tracking is based on the "phase-system switching" idea,[53–55] which was developed to solve problems of mobile phase incompatibility in LC/MS target compound analysis. An analytical column is usually connected to a trapping column in tandem mode. A switching valve is placed after the UV detector, and the flow of nonvolatile eluents is directed through the trapping column to waste. When the peak of interest elutes from the analytical column it is

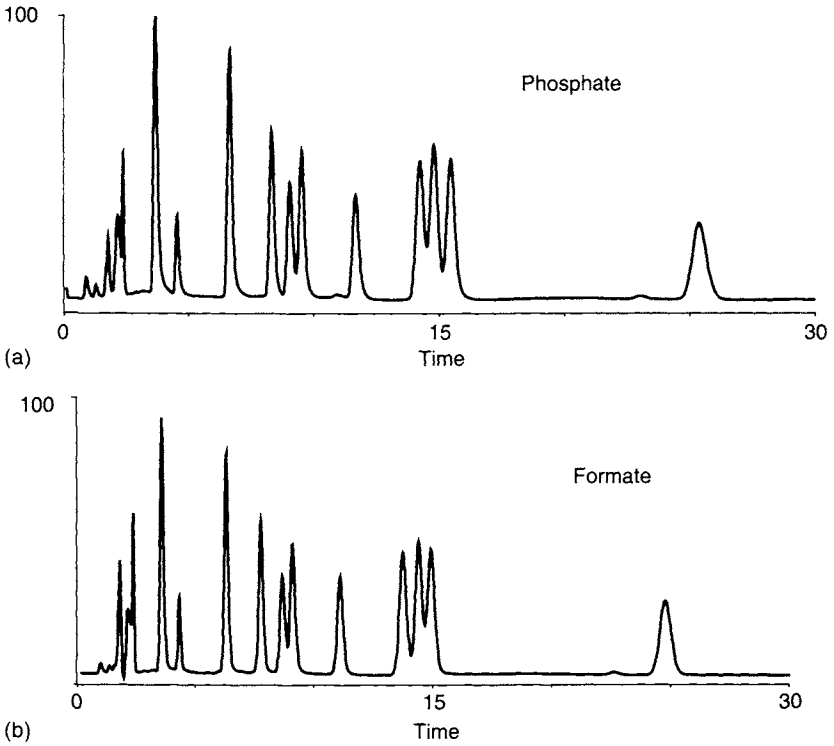


FIGURE 10 Separation of a neutraceutical mixture in phosphate- and formate-buffered mobile phases (courtesy of Waters Corp.).

switched into the trapping column where desalting takes place by pumping aqueous eluents through the column. The trapped peak can then be re-chromatographed using a mass spectrometer-friendly mobile phase for optimal mass detection. A typical setup of "on-line" peak trapping and elution is illustrated in Figure 11.

The advantage of this technique compared to the off-line fractionation approach is that it directly transfers the peak of interest for MS and MS/MS analysis to the mass spectrometer. The trapping column can be used as a concentration device if necessary. Multiple injections can be carried out on the first column to accumulate a peak of interest onto the trapping column. The trapping, however, can be a little tricky. Ermer[56] reported a successful on-line trapping and identification of an impurity at 0.1 UV-area% from a phosphate HPLC mobile phase. He suggested that the content of organic modifier used for elution of the trapped peak ought not be significantly lower than its content in the mobile phase from the nonvolatile separation. Asakawa et al.[57] reported a three-column switching configuration used in frit-FAB-MS determination of tocopherol and riboflavin. The three columns were made of the same stationary phase

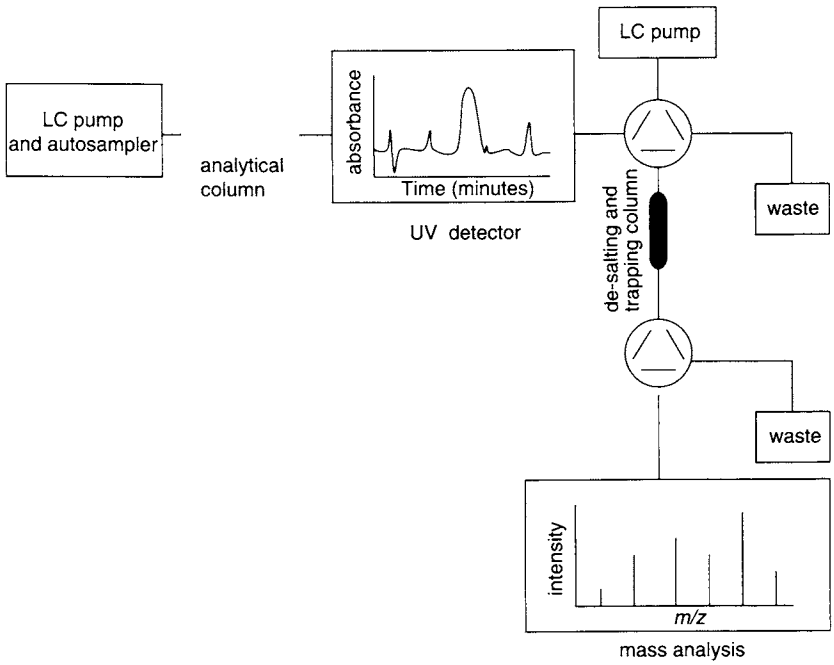


FIGURE 11 "On-line" peak trapping and elution.

and were run independent to each other. LC-1 with a conventional analytical column served to separate the compounds of interest; LC-2 and LC-3 trapped and re-chromatographed the compounds for MS analysis.

VI. DEVELOPMENT OF GENERIC LC/MS METHOD AND LC/MS DATABASE

Development and validation of HPLC methods for the assay of a drug compound and its related substances is central in pharmaceutical analysis. From pre-clinical to clinical development to final NDA submission, the methods usually go through many changes, from one set of chromatographic conditions to another set. The changes in the methods can be necessitated by changes in chemical synthesis, scale-up, modifications of drug formulations during the drug product development process, or a number of other reasons. Therefore the impurity profile of a drug substance and drug product formulation can be changing until the manufacturing processes of the drug substance and the drug product formulation are finalized. A strategic approach to dealing with this constantly changing situation was introduced by Ayrton et al.[58,59] The idea is known today as early locking-in of a "generic method." This strategy allows chemists to develop "generic HPLC methods" to use on diverse samples throughout the development lifecycle of a drug product.

A. "Generic LC/MS Method" with Basic pH Mobile Phase

A "generic HPLC method" must possess characteristics such that compounds with a wide polarity range can be chromatographed and monitored by LC/MS and LC/MS/MS. Drug degradants, such as hydroxylated derivatives of drug compounds, could be much more polar than their precursors. Therefore, a wide gradient program is usually used as the "generic HPLC method". Lee,[60] for example, suggests using a fast gradient program from 95% aqueous/5% organic to 5% aqueous/95% organic at neutral pH values (i.e., 6–7). The conditions can be refined to best suit one's need so that the desired performance is achieved. A generic gradient method generates maximum information in a short time-period rather than trying to obtain optimal resolution among all the impurity peaks. Figure 12 shows an example of how this approach was successfully used in support of formulation development of a water-soluble basic drug substance. Using a gradient more than a dozen impurity/degradation peaks were detected in a 30-min run. A high pH mobile phase was chosen to enhance the retention of the drug substance, and selectivity of the drug substance from its related substances. Several key process parameters need to be optimized during formulation development. Degradation pathways of API and the levels of degradants formed are often dependent on these parameters. The degradation peak at 3.49 min (Figure 12) was the result of *N*-dealkylation from the drug substance. The degradant is much less hydrophobic and therefore less retentive than the drug compound which elutes at a retention time of 12.25 min. On the other hand, the peaks at RT of 14.2, 15.8, 17.2 and 18.0 min were API dimerization products through either free-radical C–C coupling, the Aldol reaction, or Michael addition. There is a significant difference in the polarity of degradation products from *N*-dealkylation

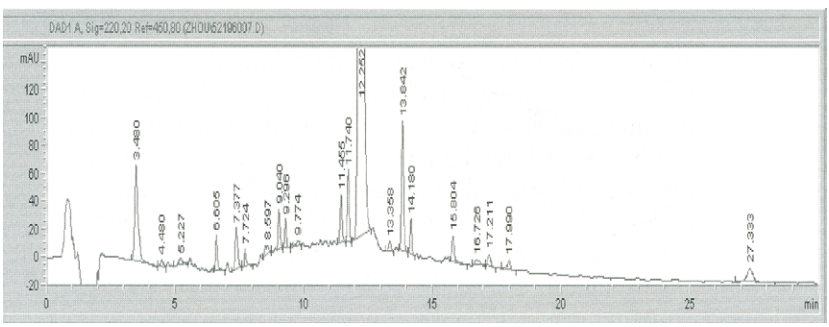


FIGURE 12 "Generic" LC/MS method with basic pH mobile phase. Column: Waters XTerra 3.5 µm MS C18, 150 x 2.1 mm at 50°C. Mobile phase: A = 0.16% of ammonium carbonate at pH 8.9 and B = acetonitrile. Linear gradient from 6% of B to 60% of B in 15 min and held for 15 min. Detection: 220 nm.

or dimerization. Figure 13 shows a total ion mass chromatogram using this "generic HPLC method". The retention time of peaks in the TIC is usually a few tenths of a minute later than their corresponding peaks in the LC/UV trace because there is a time delay for a peak to elute from the UV detector and travel to the mass detector.

B. "Generic LC/MS Method" with Acidic pH Mobile Phase

Figure 14 shows another "generic LC/MS method" used to establish the component profile for a natural product, which contains six major active components (denoted as A1, A2, A3, A4, A5, and A6). Our first trial was the use of a fast gradient with an apparent mobile phase pH of about 9, since all of these known components are acidic compounds. However, the method failed to separate A2, A3, and A4 from each other, and A6 from A5. In addition, the degradant peak D1 was not baseline resolved from the excipient peak B. When the pH of the mobile phase was lowered from 9 to 3.5, more than 35 peaks were baseline resolved in a 30-min run. The method was successfully applied to monitoring the impurity profile in different formulations. Although ESI of acidic compounds is typically performed using neutral or basic pH values, we did have adequate sensitivity at acidic pH in detecting signals for both the active components and their respective degradation products. Figure 15 illustrates a daughter ion mass spectrum for a naturally occurring impurity at a retention time of about 3.8 min. The spectrum clearly shows two

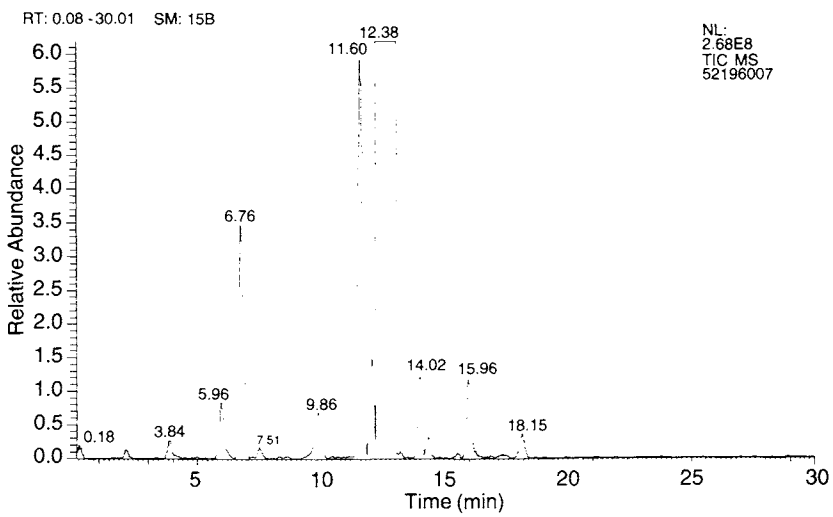


FIGURE 13 TIC of the "generic" LC/MS method with basic pH mobile phase.

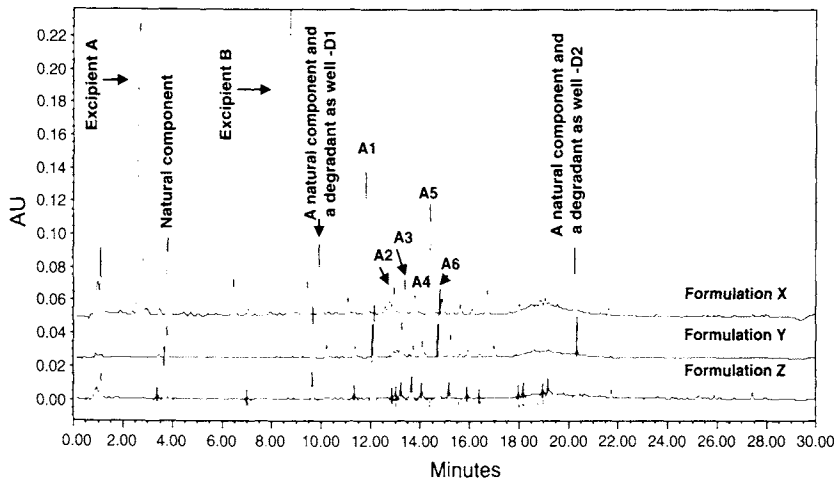


FIGURE 14 "Generic LC/MS method" using acidic pH value to monitoring of component profiles for different drug formulations. Column: Prodigy ODS(3) 3 µm, 150 x 2.0 mm. Mobile phase: A = pH 3.5 20 mM HCO_2NH_4 and B = acetonitrile. 95%A/5%B to 5%A/95%B in 15 min and hold for 15 min. Flow rate: 0.25 mL/min. Detection: 280 nm.

functional groups present in this compound. The neutral loss of 162 amu reveals that the compound bears a glucosyl group; another neutral loss of 44 amu is clearly due to the cleavage of CO_2, which resulted from the decarboxylation of the carboxylic acid group.

In summary, the strategy of using a "generic LC/MS method" in drug development provides multiple advantages. It provides a standardized starting point so that impurity profiles of drug substances and drug products can be monitored and tracked throughout the life cycle of the drug product development process. As recognized by Lee,[60] it eliminates iterative cycles of method development and refinement and provides faster project start-up.

C. Constructing a Database for Impurity Profiles

Webster's definition for "database" is "a large collection of data in a computer, organized so that it can be expanded, updated, and retrieved rapidly for various uses." An LC/MS database established for drug impurities contains multi-dimensional information such as relative retention times, UV spectra, molecular mass and substructural information. In order for the information to be updated and expanded, the methods used for information collection need to be unified. A "generic LC/MS method" allows relevant information to be collected in a consistent

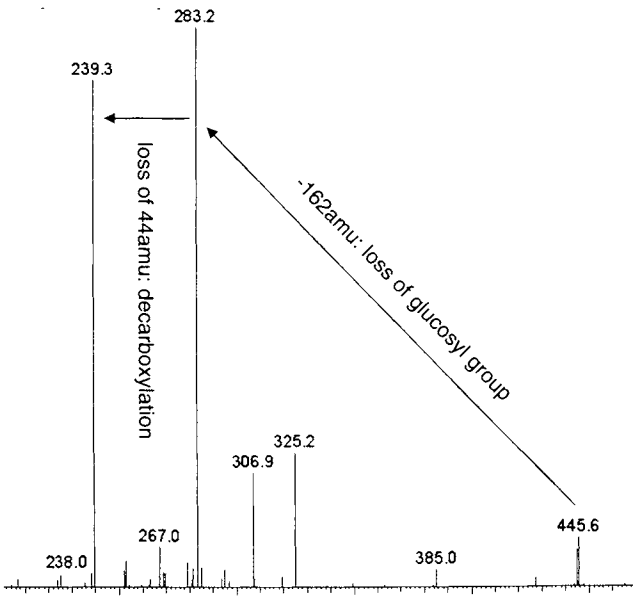


FIGURE 15 ESI/MS/MS daughter ion mass spectrum of the impurity peak at RT = 3.8 min. The impurity is a weak acidic compound, and LC/ESI MS was operated at acidic condition.

manner so that the impurity profile of one batch of drug substance or drug product can be compared to that of another batch of drug substance or drug product. This is extremely important when a drug enters the stages of clinical studies. The safety of a drug product is dependent not only on the toxicological properties of the active drug substance itself but also on the impurities therein. Qualification status established through the safety-batches ought to be maintained before further clinical studies are carried out. Analytical monitoring of impurity profiles in the drug substance and drug product plays an important role in tracking the qualification status of the API and drug product. According to the latest survey presented by the Product Quality Research Institute in its August 4–6, 2003 meeting of "Good Regulation Through Good Science" in Arlington, VA, 44% and 40% of the survey respondents said that LC/MS was the technique used for impurity characterization during phase 1 and 2 of drug development, respectively. When the development enters phase 3, 48% of survey respondents preferred isolation of the unknown impurities and complete characterization of the impurity by spectroscopic methods; 40% used LC/MS routinely to determine the content of impurities in the API and drug product.

Table 5 illustrates typical information available in an LC/MS database. More structural information such as UV spectra, parent ion and

daughter ion mass spectra can be built into the database as hotlinks tied to the identity column. For example, double-clicking on the identity column for the impurity peak at RT of 11.5 min will bring up its UV spectrum, parent ion and daughter ion mass spectra (Figure 16). Rourick et al.[61] developed an elegant predictive strategy for rapid structural identification of drug degradants using LC/MS and LC/MS/MS databases. In order to generate a collection of possible drug degradation products, an expired lot of drug substance (cefadroxil) was stressed at a variety of conditions to induce degradation. The stressed samples were chromatographed using a gradient reversed-phase HPLC method, followed by on-line LC/MS and LC/MS/MS analysis to obtain structural information for the impurities and degradants. Structural data and chromatographic behavior of 18 impurities and degradants formed under these stressed conditions were built into the database. The database contained proposed structures, relative retention time, molecular weight, and diagnostic substructures. A structural library such as the one proposed by Rourick, can provide a foundation for predicting drug stability monitoring, chemical synthesis optimization, and formulation development, etc. during the pre-clinical and developmental phases of the drug development project. The library can also provide information and predict expected degradants under typical

TABLE 5 Typical Information in an LC/MS Database

Entry	*m/z*	RT (min)	UV shift?	Identity
1	288	3.47	No	Dealkylation degradation product
2	360	3.47	No	Hydroxy-substituted product
3	344	5.23	No	*N*-oxide
4	328	6.59	No	Geoisomer, process impurity
	328	12.25		API
5	669	7.36	Yes	Aldol adducts between API and 10-oxo-API
6	669	11.76	Yes	
7	671	9.04	No	Aldol adducts between API and 10-hydroxy-API
8	671	13.85	No	
9	326	11.47	Yes	Quinone like API
10	342	Shoulder peak	N/A	Di-keto
11	653	14.17	Yes	Bis-coupling API (2,2' C–C coupling, or ether linkage)
12	653	18.00	Yes	
13	655	15.82	No	Aldol adducts (5,6' , or 7,6' C–C coupling)
14	655	17.23	No	

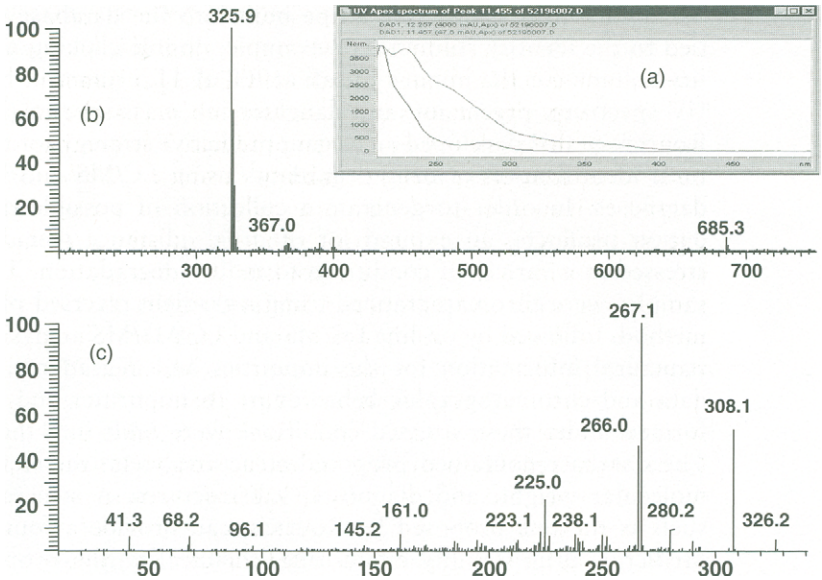


FIGURE 16 (a) UV spectrum, (b) parent ion and (c) daughter ion mass spectra of an impurity peak can be built into an LC/MS database.

drug processing and storage conditions, and sometimes drug metabolites under physiological conditions.

Xu et al.[62] also generated a degradant database for sumatriptan succinate by subjecting the drug substance to different stressed conditions followed by subsequent structure elucidation studies using LC/MS and LC/MS/MS. Sumatriptan succinate is a serotonin agonist effective in the acute treatment of migraine headaches. The study concluded that the drug was stable on exposure of acid, base, oxidation, and UV radiation at ambient conditions.

One of the key elements in such structural databases is the MS/MS daughter ion mass spectra of impurities. When impurity standards are not available, the spectra can serve as reference in prediction and identification of unknown degradants. Nicolas and Scholz[63] developed a method to assess and test the feasibility of using LC/MS/MS daughter ion mass spectra as "fingerprints" for structure identification. The fingerprints included a precursor ion mass, and at least three daughter ion masses. The authors generated MS/MS fingerprints for a drug substance and several of its impurities using either available standards or on-line LC/MS/MS analysis of a drug substance lot used in the earliest safety study. Several subsequent lots of drug substance were examined and the fingerprints of the impurities were obtained. When the same collision energy was used in the analysis of the same impurity peak, its MS/MS fingerprint matched

very well with that obtained for the impurity from the safety lot. Nicolas and Scholz were successful even when they studied impurities at very low levels, such as 0.01 UV area percent of the drug substance.

Drug impurity databases, as discussed above, can be generated based on stressed drug substances or drug products. The databases can also be built using long-term storage stability samples. While various forced degradation studies, in which a drug substance or a drug dosage form is exposed to environmental factors such as acid, base, heat, light, oxidant, etc. can provide a diversity of degradant structures, overstressing of the sample can occur and lead to degradation profiles that are not representative of real storage conditions and perhaps not relevant for stability monitoring. Volk et al.[64] reported their LC/MS and LC/MS/MS investigation results on an aged sample of butorphanol tartrate that was stored in the dark for 170 weeks at 30°C. The investigation used the same analytical approach, i.e., acquiring molecular weights for the impurities during LC/MS profiling followed by LC/MS/MS substructural analysis.

Once a database is established, it is made available to other laboratories through the company's secured intranet, so that the information therein can be updated, retrieved and reviewed. The resulting structural library can be referenced throughout the lifetime of the drug for rapid identification of impurities, degradants, and metabolites.

VII. MS-ASSISTED HPLC METHOD DEVELOPMENT AND VALIDATION

A. Peak Purity Assessment

HPLC with UV detection is the analytical technique most often used for the assay of the active pharmaceutical ingredient (API) and determination of impurities in API and drug products. HPLC methods designed for assay of API, and the determination of impurities must be stability-indicating. A minimum requirement in chromatographic method development is to ensure specificity of the method, i.e., to achieve a set of chromatographic conditions in which the analyte peak is free from interference or contamination by other components of the formulation. The suitability of an HPLC method must be demonstrated in the validation step. For example, specificity of the method must be demonstrated to show that the API peak is separated from impurities; or that a degradant peak is well resolved from other degradants or synthetic impurities.

Diode-array UV detectors (DAD) are generally used to conduct peak-purity examination. The purity check is based on the comparison and matching of UV spectra at various points across the HPLC peak to that at the apex of the peak. The inherent disadvantage of using DAD for peak purity investigations is that it can only reveal spectral homogeneity, but not chemical homogeneity, across a chromatographic peak. This approach assumes that the UV spectrum obtained at the apex is free of

any co-eluting impurities, and that the UV spectra of impurities are at least slightly different from those of the API. However, many impurities such as degradants and synthetic impurities are structurally related to the API, and contain in their structure very similar chromophores, making purity assessment based solely on DAD data not reliable. Therefore, an LC peak with homogeneous UV-spectral characteristics does not necessarily prove chemical purity of the peak. Other concerns in using DAD alone may include concerns that analytes many not contain chromophores, analytes may be present at very low concentration levels, etc.

Coupling a mass spectrometer to a liquid chromatograph brings a new dimension to specificity studies. Since a mass spectrometer separates compounds by their respective mass-to-charge ratios, any difference in the *m/z* values between the impurities and the drug substance will allow an unambiguous detection regardless of similarities in their UV spectra. Therefore chromatographic co-eluting components will be separated in MS as long as their *m/z* values are different.

Figure 17 shows an example where LC/DAD-UV versus LC/MS is contrasted in the establishment of chromatographic peak purity. Comparison of the UV spectra taken on the upslope, apex, and downslope of the peak reveals that the peak is spectrally nonhomogeneous. However, the method cannot address questions as to how many components are co-eluting, and structural information for these components is unavailable. LC/MS, on the other hand, gives unequivocal peak identification by comparing mass spectra extracted from the same three locations of the chromatographic peak. The mass spectrum extracted from the onset of the chromatographic peak shows a single chemical entity with mass-to-charge ratio of 309. A second ion with *m/z* of 287 is seen in the spectrum extracted from the apex of the peak; the relative intensity of this second ion vs. the ion at *m/z* of 309 increases in the spectrum on the offset of the peak.

Bryant et al.[65] have reported the suitability of using ESI-MS for assessment of peak homogeneity. The HPLC system was designed to intentionally co-elute a number of impurities within the peak of the drug substance. Mass spectra across an HPLC peak were summed followed by background subtraction to examine the co-eluting impurities. The co-eluting impurities can be rapidly detected to a level of 0.02% of the peak of the drug substance. The method is shown to be superior to the UV method in that compounds with identical and similar UV spectra can be distinguished.

B. Peak Tracking in Method Development

LC/UV detection, because of its sensitivity, robustness and precision, is the most commonly used technique for the detection and quantitation

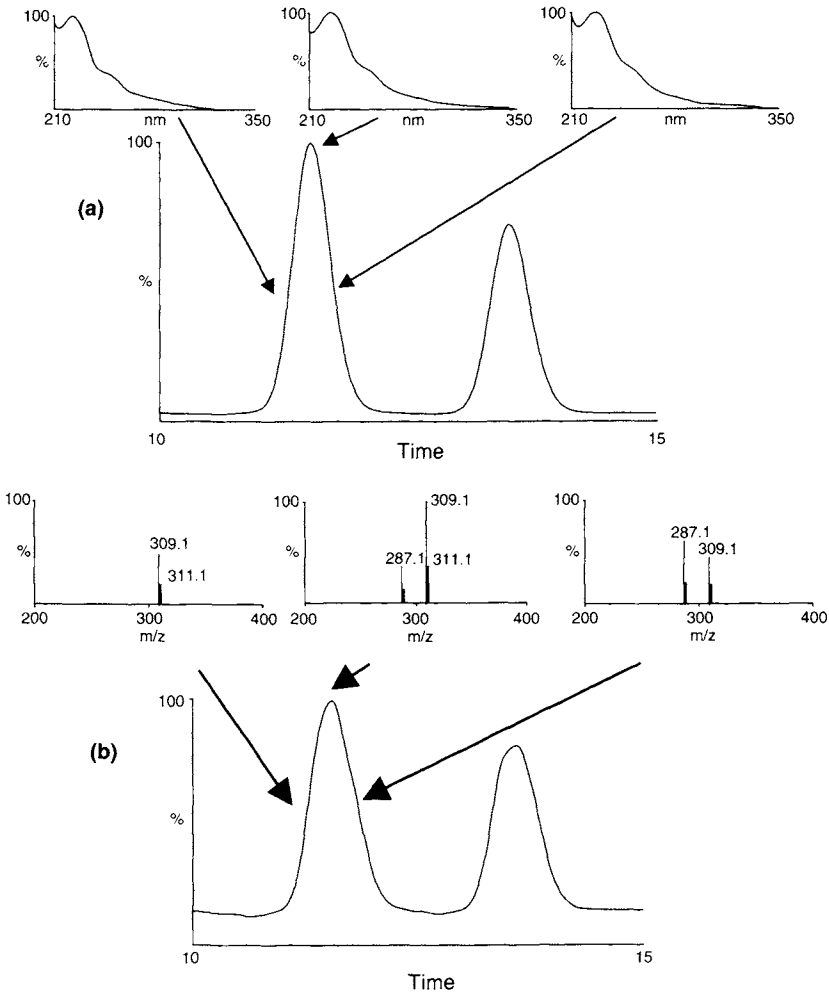


FIGURE 17 Peak purity detection using (a) LC/DAD-UV vs. (b) LC/MS (courtesy of Waters Corp.).

of impurities in drug substances and drug products. As discussed in Section VI, LC/UV methods designed for the determination of impurities usually undergo many changes from one set of chromatographic conditions to another during the drug product development process. Each such change may result in different elution order of impurity peaks. Figure 18 shows two chromatograms with (a) corresponding to an acetonitrile-based mobile phase and (b) corresponding to a methanol-based mobile phase. During early drug development, reference standards for many of the drug impurities may not be available. Correlation for the group of peaks at RT from 12 to 15 min could not have been established

if the only information available was the percent of peak area and the UV spectra of the peaks. Conversely, full scan LC/MS allowed simultaneous detection and molecular mass determination of these three peaks in both mobile phase systems. Figure 19 presents full scan LC/MS results for impurities separated using both chromatographic methods. The molecular ions, inarguably, provide adequate information to allow peak correlation.

C. Development of an HPLC Method to Account for Mass Balance

Execution of stability programs for drug substances and dosage forms is a central focus during the development of drug products. One of the objectives that needs to be achieved from the stability data is to establish a mass balance equation for the API. A viable pharmaceutical formulation is expected not only to deliver desired pharmacokinetics but also to demonstrate good stability behavior for the API under recommended storage conditions. However it is inevitable for the API to experience some degree of degradation when the formulation is exposed to environmental factors such as heat, humidity and UV light. The degradation of API in formulations can be very complex and sometimes unpredictable. It is not unusual for the decrease in the assay value not to be accompanied by a proportional increase of degradation products. The

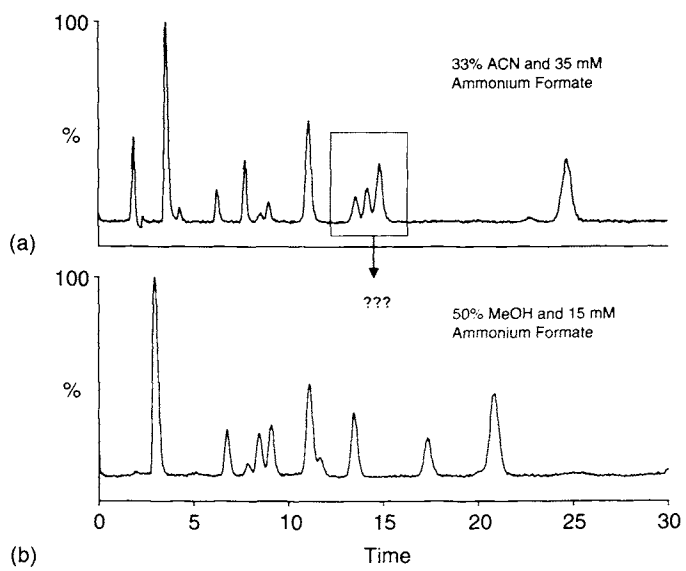


FIGURE 18 **A challenging case in which peak correlation between two LC methods cannot be achieved based on the percent of peak area and the UV spectra of the peaks (courtesy of Waters Corp.).**

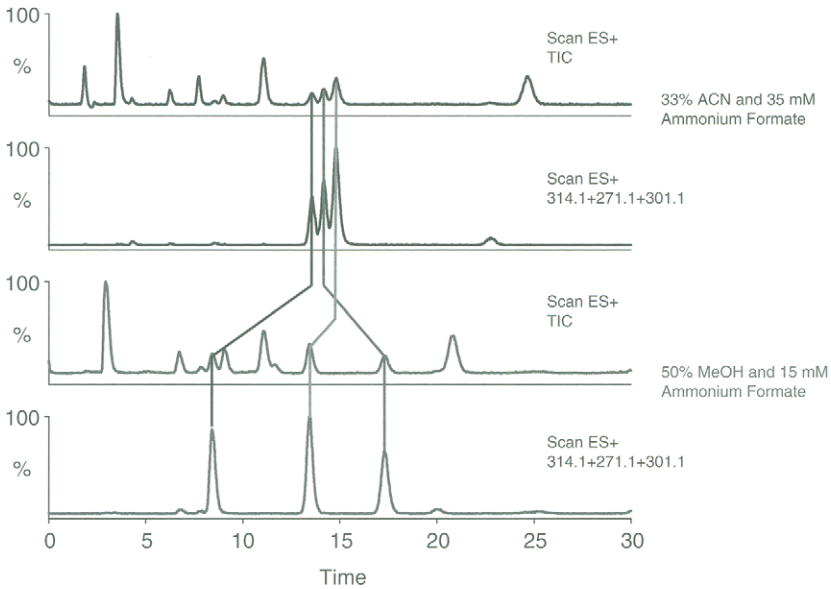


FIGURE 19 Detection of molecular ions from full scan LC/MS snap the peak correlation between two chromatographic methods (courtesy of Waters Corp.).

detection limit of LC and the change in the UV chromophores of degradation products make the justification of mass balance a very challenging task during drug development.

The degradation of the API can be of unimolecular reactions such as dehydration, epimerization of API. More often encountered are bimolecular reactions such as oxidation, hydrolysis, and dimerization of the API. Reactions of API with excipients and packaging components are also possible. In some cases, a degradant derived from multiple-stage degradation processes can still react with the API. Ginsburg et al.[66] reported a degradation product in a parenteral formulation as a result of a reaction between the API and an oxidation product of an excipient. Butamben (butyl *p*-amino-benzoate) was formulated to provide local anesthesia for the treatment of chronic pain. The formulation was found to undergo discoloration when the formulation was stored at ambient conditions. The compound responsible for the discoloration was isolated and identified by positive FAB-MS and $^{1}H/^{13}C$ NMR experiments as an oxalamidine. The oxalamidine was the condensation product of oxalic acid and four equivalences of butamben. Oxalic acid, according to Ginsburg et al.,[66] was the oxidation product of poly(ethylene) or/and polysorbate 80 – both were excipients in the formulation.

Direct infusion mass spectrometry is a popular technique used by medicinal chemists to check the molecular mass of target compounds. In

the author's laboratory, it is often used to build an "impurities inventory" for a stressed drug substance or drug formulation. In order to establish mass balance for the API in a drug formulation, all the degradation peaks must elute from the HPLC column and be detected at the chosen wavelength. The polarity and solubility of some degradants can be drastically different from that of the API. Poor recovery of impurities and degradation products during sample preparation, combined with inappropriate choices of organic strength and pH values in the mobile phase can make impurities and degradation products undetectable. If one or more of the drug degradants were not detected in an HPLC chromatogram, one would likely experience an unexplainable mass loss.

Forced degradation of a drug substance or a drug product is usually conducted to demonstrate method specificity when developing a stability-indicating chromatographic method. Forced degradation can be carried out using different combinations of heat, humidity, light, acid, base, or oxidant. These studies also provide information about what degradation products could form during storage. Impurity profiling studies using MS and MS/MS approaches on the stressed samples can lead to an inventory of impurities. Data built in this "inventory" provides information on not only the degradation pathways but also on chemical markers to ensure the successful development of methods for assay and impurities determination.

Although ionization suppression of minor components can occur during the analysis of concentrated solutions of API, the ultimate resolving power and accurate mass measurement of the FTMS can still be the best choice to generate qualitative impurity profiles. Direct infusion of a sample allows the detection of all ionizable constituents. It also bypasses the LC separation, and avoids selective loss of components at extreme ends of the polarity scale. In one instance, during formulation development, up to a 20% loss of API was observed after storage for 6 months at 40°C/75% RH. A reversed-phase LC method was used for the assay of the API. The mobile phase consisted of 80% of 20 mM HCO_2NH_4 at pH 3.2 and 20% of CH_3OH and CH_3CN in a 1:1 ratio. A total of 5% of three degradation products was observed using these HPLC conditions. However, a significant 15% loss of drug compound could not be accounted for. A stressed sample, heated at 80°C in an oven chamber saturated with moisture, was analyzed by the direct infusion into it of a Bruker Apex II 4.7 Tesla FTMS. Figure 20 shows the mass spectrum for the stressed sample. Eighteen likely degradation products were observed. Based on their exact masses and chemical reactivity of the API, structures for 14 of these degradants were proposed (Table 6). An improved HPLC assay method was developed based on the information obtained from the direct infusion FTMS results. Figure 21 presents a comparison of the original assay method to the improved method. One dominant degradation product was identified as the result of dealkylation of the drug

molecule. The dealkylated degradant was much more hydrophilic, and as a result was eluting in the solvent front in the original HPLC method. A Waters XTerra column was chosen to replace the originally used Symmetry column because polar compounds are more retained with XTerra columns. Other adjustments in order to retain this degradant included lowering the organic strength at the beginning of the chromatographic run, and increasing the pH value of the mobile phase from 3.5 to 9. Many of the degradation products were identified as dimerized compounds; some of them did not elute from the column in the original, isocratic method. A gradient elution program was therefore used to end the chromatographic run with 60% of acetonitrile, and simultaneously the temperature for the column was raised to 50°C from 40°C. With all the modifications, seven more degradant peaks were detected, which brought the mass balance to about 98% when the quantitative levels of impurities were expressed as a percentage of the peak area of the API peak.

VIII. IMPURITY PROFILING FOR DRUG SUBSTANCES AND PHARMACEUTICAL PRODUCTS

Toxicity and pharmacological effects of impurities in a pharmaceutical formulation are monitored in a variety of safety and clinical studies.

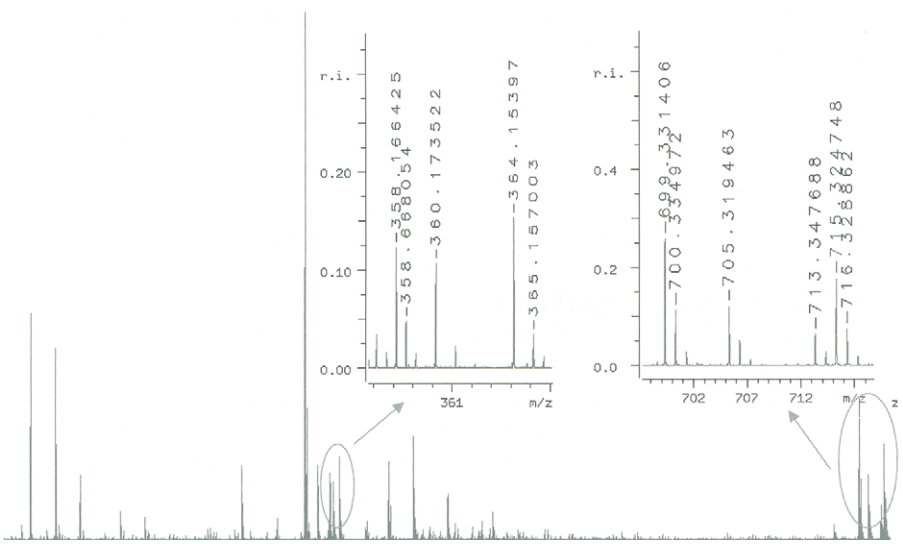


FIGURE 20 FTMS Spectrum of a stressed sample: the API signal appeared at *m/z* of 342, 18 degradants were detected.

TABLE 6 Direct Infusion FTMS Results on a Stressed Solid Dosage Formulation

Peak#	Experimental	Theoretical	Error (ppm)	Structural information
1	149.02334	149.02332	0.16	Environment contaminant—phthalate
2	288.12289	288.12303	0.47	Degradant resulted from *N*-dealkylation
3	324.15956	324.15942	0.43	A likely dehydration degradant
4	340.15488	340.15433	1.62	Quinone-type degradant
5	356.14842	356.14925	2.33	Two steps of oxidation to give rise to keto-degradant
6	358.16468	358.16490	0.61	Hydroxylated degradant
7	360.18082	360.18055	0.75	Addition of water
8	388.17496	N/A	N/A	?
9	390.15434	N/A	N/A	?
10	396.21659	396.21693	0.86	*Trans*-alkylation degradant
11	406.18485	N/A	N/A	?
12	665.32117	665.32213	1.44	Aldol product of API
13	681.31830	681.31704	1.85	Free radical dimerization of API; pesudo product of API
14	683.33283	683.33269	0.20	Aldol adduct of API
15	679.30221	679.30139	1.21	Aldol product of API and *m/z* 356
16	695.29974	695.29631	4.94	Free radical coupling of API and *m/z* 356
17	697.31573	697.31196	5.41	Aldol product of API and m/z 356
18	705.31765	N/A	N/A	?
19	713.30745	713.30687	0.81	Free radical coupling of *m/z* 356 and *m/z* 360

The degradation and impurity profiles are critical to the correct safety and potency assessment of the drug product for clinical trials. LC/MS and LC/MS/MS have given an impetus to both qualitative and quantitative analyses of drug impurities and degradants.[67–74] When fragmentation information in combination with molecular weight is not sufficient to differentiate between isomeric and isobaric structures, other techniques are sometimes used, such as the rapidly advancing, on-line coupling LC-NMR for structural proposals.[75,76] The applications of LC/MS and LC/MS/MS for pharmaceutical development are discussed in a number of review articles.[77–81] There are similarities and differences in the impurity profiles in a drug substance and the drug product. Therefore, the qualification and the strategy for their identification should be treated accordingly. For drug substances, impurities are subdivided into

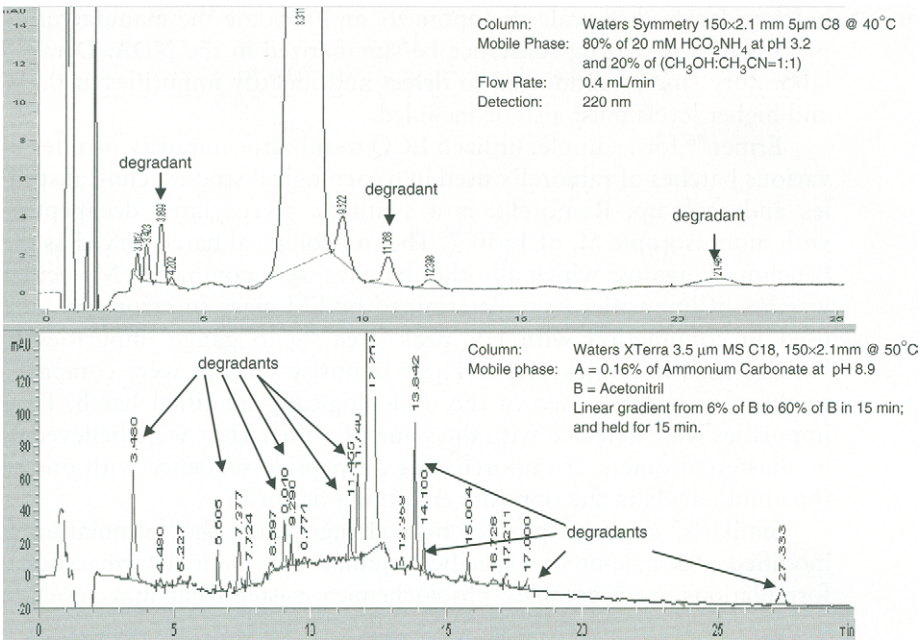


FIGURE 21 **(a) An isocratic reversed-phase HPLC method developed for assay, with which 15% of mass balance was not accountable. (b) An improved gradient HPLC assay method based on infusion FTMS results, with which seven more degradation product peaks can be detected.**

inorganic impurities, organic impurities, and residual solvents. Organic impurities may arise during the manufacturing process and/or storage of the drug substance. Typical impurities include synthetic impurities such as starting materials, by-products, intermediates, ligands and catalysts. Typical degradation products can be oxidation products, hydrolysis products, epimerization products and dimerization products of the drug molecule. For drug products, only the impurities formed by the degradation of the active pharmaceutical ingredient and by reaction of the API with an excipient and/or some component of the container closure system have to be taken into consideration. However, the complexity of the matrix in pharmaceutical formulations poses a significant challenge for LC/MS analysis. Many excipients used in the formulation can cause detection problems. For example, signals from PEG, as discussed in Section IV. E. and illustrated in Figure 8, can dominate the mass chromatogram.

During the course of chemical development, impurity profiles in drug substances may change due to changes in synthetic route and changes in the size of the batch. ICH guidelines for Impurities in New Drug Substances (ICH Q3A), require that impurity test results for

batches designed to scale-up, optimize, and validate the manufacturing process of a new drug substance be summarized in the NDA. Detailed laboratory studies conducted to detect and identify impurities at 0.1% and higher levels must also be included.

Ermer,[56] for example, utilized LCQ to monitor impurity profiles of various batches of ramorelix used in toxicological studies, clinical studies and scale-up. Ramorelix is a synthetic glycosylated decapeptide with monoisotopic M_r of 1530.7. The toxicological batch served as the benchmark against which all other batches were compared. Molecular weights of impurities were determined by ESI mass spectrometry, and used in conjunction with UV peak area % to gauge impurities in batches used in clinical trials. These impurity profiles were compared to those of batches used in the toxicologically qualified batch. Four impurities were detected with the same M_r value. They were believed to be diastereoisomers of ramorelix, i.e., a peptide sequence with one of the amino acids in the opposite enantiomeric form.

Similarly, impurity profiles may change when the formulation is modified or a scale-up of a specific formulation is made. Pharmaceutical formulations are a complex physiochemical system that may result in impurities due to reactions between API and pharmaceutical excipients and/or packaging materials. In some cases, degradants that were generated by multiple-step degradation pathways can still react with the API leading to the formation of degradants that can be difficult to identify.

In a package screening study, a low level degradant of famotidine was detected when stored in child-resistant foil pouches. The identity of the impurity was investigated using HPLC/APCI mass spectrometry.[82] The molecular weight of the impurity was reported as 12 amu higher than that of famotidine, indicating the insertion of one C atom to the drug compound. Detailed substructural analysis by comparison of the fragmentation pattern with that of famotidine suggested that the carbon insertion occurred at the *N*-(aminosulphonyl)-propanimid-amide end of famotidine. When famotidine was exposed to a source of formaldehyde, a degradation product with the same molecular weight was formed. Unfortunately, there was no discussion in the paper as to whether the synthesized compound was the one detected in the child-resistant packaged drug product.

A distinction needs to be made between identification and qualification of degradation products. Identification means that the structure has been elucidated, while qualification is defined as the process of acquiring and evaluating data to establish the biological safety of an individual impurity or a given impurity profile. The analytical method must be sensitive enough to quantitate known impurities at the specified limits and unknown impurities at the levels required by the ICH guidelines. Successful identification of drug impurities using LC/MS and LC/MS/MS always relies on the generation of molecular weight,

substructural information, UV spectrum, and the chemical reactivity of the drug compound.

A. Determination of Molecular Mass

Protonated and cationized species are the most commonly detected ions using the ES process if a positive ionization mode is selected. Protonation is a result of the addition of proton(s) to a neutral molecule; for every proton added, a net charge of +1 will result. Similarly, cationization is due to the addition of cation(s) to a neutral molecule. Detection of cationized ions can be useful in the determination of molecular mass of unknown analytes.[83] If ESI is operated under negative ion mode, deprotonated ions will usually be the most dominant ions. For either operation mode, solvent adducts of the protonated/deprotonated ions are frequently detected in ESI/MS mass spectra.

Assignment of various ions in an ESI/MS spectrum is usually straightforward. Although high levels of chemical noise in the total ion chromatogram are inherent in the ES process, many algorithms are available in commercial instruments which allow background subtraction to correct for part of the chemical noise. The chemical noise results from a number of sources, including the LC mobile phase and buffers that give rise to high contributions in the signal. Figures 22(a) and (b) show LC/UV and ESI–TIC traces, respectively. The mass spectrometric analysis was conducted on a Finnigan MAT TSQ 7000 with ESI positive ion monitoring. Figure 22(c) is the extracted mass spectrum for the peak at retention time of 25.35 min without background subtraction, while Figure 22(d) shows the mass spectrum for the same peak with background subtraction. Despite the noisy appearance of the TIC trace, individual mass spectra, as evident in Figure 22(c) and (d), obtained from ESI LC/MS are generally of good quality.

The first step in establishing an impurity profile is to extract mass spectra at the positions of impurity peaks in the corresponding UV chromatogram. A profile of selected ion mass chromatograms can be obtained by extracting ions with relevant mass-to-charge ratios and compared to the UV trace. Assignment of various ions will lead to the determination of molecular masses of impurities. The ions observed in Figure 22(c) and (d) can be designated in Table 7. One of the characteristics in the ESI mass spectrum is that cationized ions as well as solvent adducts of protonated pseudo molecular ions are frequently observed. Since the protonated pseudo molecular ion was detected with mass-to-charge ratio of 344, the molecular mass for the impurity peak at RT=25.35 min is 343 amu. Molecular masses of impurities may reveal rich structural information. For example, an

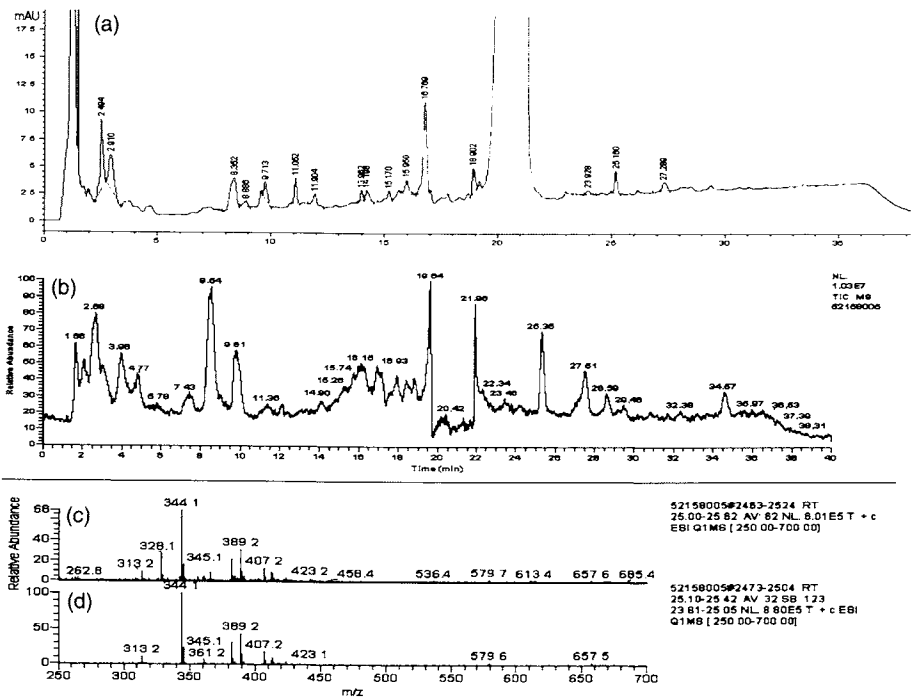


FIGURE 22 (a) HPLC/UV chromatogram. (b) ESI LC/MS total ion chromatogram. (c) extracted mass spectrum for RT = 25.35 min peak without background subtraction. (d) Extracted mass spectrum for the same peak with background subtraction.

TABLE 7 Assignment of Ions Detected in ESI Parent Ion Mass Spectrum

Ions (*m/z*)	Assignment of ions
313	Chemical noise or thermally degradated ion
328	Chemical noise
344	$M + H^+$, protonated pseudo molecular ion
345	"A+1" isotope of $M + H^-$
361	$M + NH_4^-$, ammoniated pseudo molecular ion
382	$M + K^+$, potassiated pseudo molecular ion
389	$M + C_2H_5NH_2 + H^+$, ethyl amine adduct of the protonated pseudo molecular ion
407	$M + HCOONH_4 + H^-$, ammonium formate adduct of the protonated pseudo molecular ion

increment of 16 amu in the molecular mass of a degradant relative to the drug compound usually suggests oxidation of the drug compound to form hydroxy or *N*-oxide compounds; an increment of 14 amu

suggests the formation of ketones via dual-stage oxidation of the drug compound.

B. Determination of Exact Mass and Elemental Composition

Determination of elemental compositions is an important step towards the structural elucidation of drug impurities. Elemental composition of an unknown compound can only be derived from the measurement of its exact mass. In combination with the knowledge of the physiological or chemical process, elemental compositions alone may be enough to propose a correct structure.

There are three kinds of mass spectrometers capable of generating high-resolution mass spectra and determining accurate mass: the TOF mass spectrometers or the hybrid quadrupole time-of-flight (Q-TOF) mass spectrometers, Fourier transform ion cyclotron resonance (FTICR) mass spectrometers and the double-focusing sector mass spectrometers. In order to achieve high accuracy in mass measurement, mass spectrometers must be calibrated with calibrants of known exact masses. With internal or external calibration, good mass measurement can be achieved. For example, FTMS can generate mass measurement with accuracy of less than 1 ppm in mass error between the experimentally determined value and the theoretical exact mass.

All elements have uniquely assigned masses using a reference standard of ^{12}C as 12.00000. Chemical compounds with unique elemental compositions will, therefore, have a distinguishing exact mass. Computer programs are available to calculate possible elemental compositions for the measured accurate mass. Many different elemental combinations may exist for a molecule with a specific accurate mass. The number of possible compositions increases with increasing mass and the increasing number of elements expected to be present in a molecule. The more accurate the measured mass, the smaller the number of possible elemental compositions that will be derived. As important as mass accuracy, the isotopic pattern in a full scan high-resolution mass spectrum can be used as a fingerprint to help derive the elemental composition of the unknown. The abundance of isotope peaks is governed by the natural occurrence of the elements in the ions. The presence of "A+2" elements such as Cl, Br, Si and S can usually be recognized from the higher-than-normal intensity of the "A+2" peak. The maximum number of carbon atoms can be determined by dividing the peak ratio of "A+1"/"A" by the natural abundance of ^{13}C - 0.011.

Since FTMS provides high resolution and high mass accuracy, it enables detailed structural investigation for small molecules and proteins as well. There has been a publication[84] examining the effectiveness of accurate mass measurements in minimizing false protein

matches by varying the mass error allowed in the search over a range from 2 to 500 ppm. The report evaluated the utility of accurate mass tags by calculating the number of possible enzymatically digested polypeptide fragments for all predictive proteins from *C. elegans*. Out of 19 100 predicted proteins, or 918 655 possible tryptic fragments, approximately 60% of the peptides with molecular mass of 2000 Da or less have unique masses at a mass measurement accuracy of 0.1 ppm; at an accuracy of 1 ppm, this value decreases to 40%. For the peptides with mass of 2500 Da and higher, more than 80% have unique masses at the sub-ppm error level.

Eckers et al.[85] used on-line coupled reversed-phase LC to a Q-TOF mass spectrometer, and have successfully identified trace impurities in cimetidine. In an earlier paper published by Haskins et al.,[86] they successfully identified four chromatographically unresolved reaction by-products in cimetidine, using LC-FTMS. Another example using fast LC coupled with a Q-TOF mass spectrometer was the identification of cimetidine-related drug substance impurities reported by Lee et al.[87] The exact masses for six impurities were determined with an experimental error of less than 3.1 ppm.

A case study of the identification of a counterfeit drug molecule is discussed in Section IX. C. This is a step-by-step discussion of the experimental procedure using FTMS to address this important issue. After the exact mass of the unknown compound has been determined, the next step is to derive its elemental composition. The minimum and maximum number of expected atoms present in the compound must be specified in the search criteria to allow the computer program to calculate possible elemental compositions. A unique fit of only one elemental composition is rarely obtained. The number of possible compositions increases with the increasing number of elements present and with increasing mass. However, other information, such as the number of double bond equivalency and the isotopic distribution of the parent ion mass spectrum, can be used to reduce the possible elemental compositions to a reasonable number. Further discussion can be found in Section IX. C.

C. Structural Identification and Elucidation

Traditional methodologies for structural identification of trace level impurities in drug substances/products usually involve fractionation of each impurities using a scaled-up analytical chromatographic method, followed by off-line spectroscopic analysis. Coupling of HPLC separation and electrospray mass spectrometry allows on-line acquisition of full scan mass spectra and generation of tandem mass spectrometric data. LC/ESI MS has revolutionized trace analysis for qualitative and quantitative studies in pharmaceutical analysis.

1. Use of the Drug Molecule as a Structural Template

The interpretation of the daughter ion mass spectra is often hampered by a lack of knowledge of the fragmentation rules. ESI MS/MS spectra are often characterized by frequent and complicated hydrogen-shifts. Hence, an alternative method for the elucidation of mass spectra is vital. Fortuitously, when a drug compound degrades, most of its degradants would be expected to retain a major portion of the substructure of the parent drug compound. A substructural analysis based on specific product ions and neutral losses from the drug compound can serve as "templates" for the interpretation of the structures of unknown degradants. This is a widely used method[88,89] to help identify the structures of unknown degradation products. Precursor ions, usually protonated pseudo molecular ions, are selected and subjected to low-energy CID in the presence of collision gas. The dissociations produce fragmented ions that are recorded in the MS/MS spectrum. The comparison of MS/MS patterns of unknown degradants to those of the drug compounds is likely to reveal similarities and the differences. Common product ions and neutral losses observed in the drug compound and the degradants suggest common substructures, and the differences indicative of the modifications of substructures and locations of such modifications have occurred.

Figures 23(a) and (b) show daughter ion mass spectra of a drug molecule and one of its impurities. The spectra were acquired using a Finnigan MAT TSQ 7000 interfaced to an Agilent 1090 LC, operated in the ES negative ion mode. The *m/z* of 861 was the de-protonated pseudo molecular ion. The base peak, with m/z of 699, was 162 amu lower than that of the deprotonated pseudo molecular ion. The loss of 162 amu was due to deglucosylated fragmentation, a signature loss that is consistent with the structure of the drug molecule, which bears the glucosyl functionality. Another abundant daughter ion was observed at *m/z* of 817, corresponding to a loss of 44 amu from the parent molecular ion. This loss was indicative of decarboxylation from the drug molecule. This type of fragmentation pattern was expected because the drug molecule contains a carboxylic acid moiety. The impurity, however, did not show the loss of 162 amu. Instead, a loss of 324 amu was detected as the predominant fragmentation ion. The loss of 324 amu is consistent with the molecular weight of lactose, which suggests that this impurity contains a lactosyl functionality.

2. Hydrogen/Deuterium Exchange Experiment

Hydrogen/deuterium (H/D) exchange experiments can provide critical information for structure elucidation. The use of H/D exchange is a well-accepted protocol in the field of proteomics for identification of proteins.[90,91] The generation of proteolytic peptide mapping for protein identification has been reported. This involves labeling selected amino acids with ^{13}C, ^{15}N, or ^{2}H and incorporating them into proteins during cell culture.[92] Each of the labeled amino acids carries a defined change in

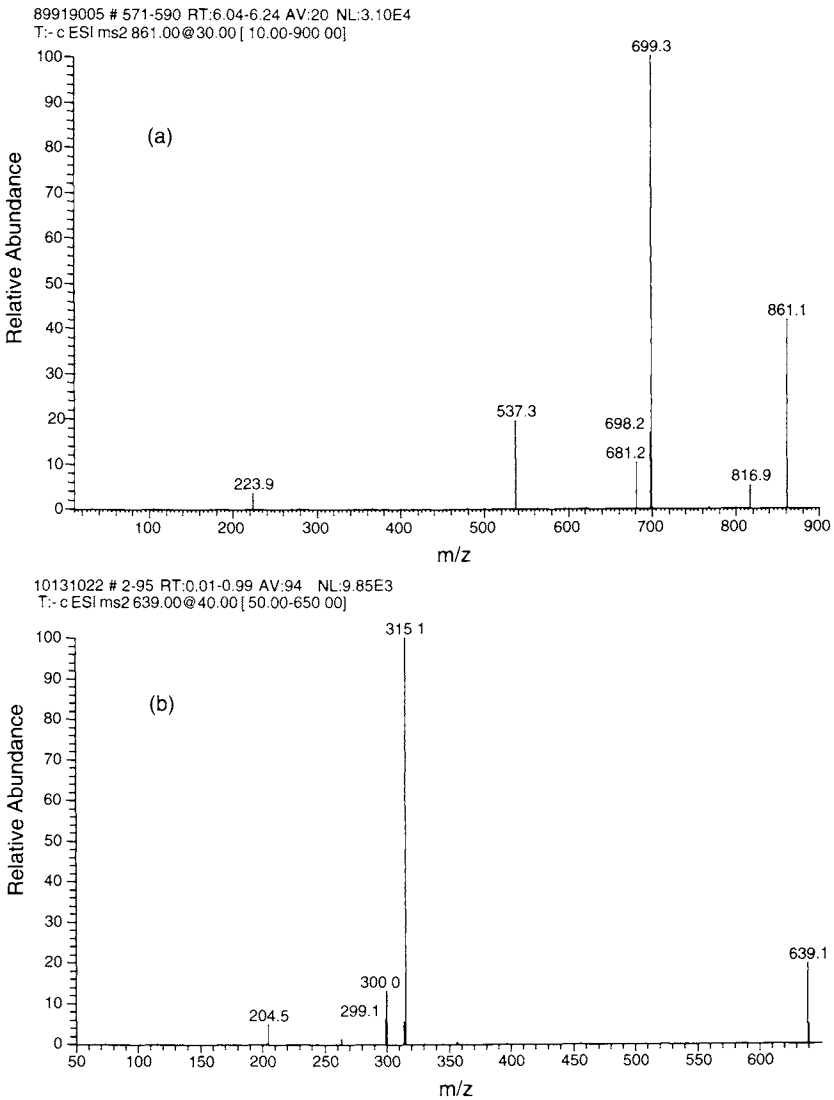


FIGURE 23 Comparison of daughter ion mass spectra of drug molecule (a) and its impurity (b).

mass, and a change in the appearance of the monoisotopic distribution pattern in the mass spectrum. These changes in the mass spectrum permit the isotopically labeled peptides to be distinguished from other peptides after proteolysis of the protein.

The application of H/D exchange experiments in the identification of small molecules is of great importance as well, and has also been reported.[93,94] The experiment is usually carried out using deuterium oxide

as a source of deuterium for incorporation into the unknown compound. The change in mass for the unknown in deuterium oxide (compared to water as the solvent) is indicative of the number of exchangeable protons such as hydroxyl, amine, amide, thiol, and carboxylic acid groups present in the unknown compound. A shift of "n" amu units in the mass to charge value when mass spectrometer is operated under positive ion mode suggests "$n-1$" exchangeable protons because the proton carrying the charge of the ion is also shifted. On the other hand, a shift of "n" in negative ionization mode reveals that "$n+1$" exchangeable protons are present in the unknown compound. In our laboratory, the technique is frequently employed to aid in the identification of drug impurities. For example, an unknown impurity peak was observed in a natural product during the development of an HPLC assay method. LC-ESI negative ion mass spectrometric analysis confirmed the nominal mass of this impurity as 480 Da. The daughter ion mass spectrum of this unknown compound was acquired on a Bruker Apex II 4.7 Tesla FTMS. As seen in Figure 24(a),

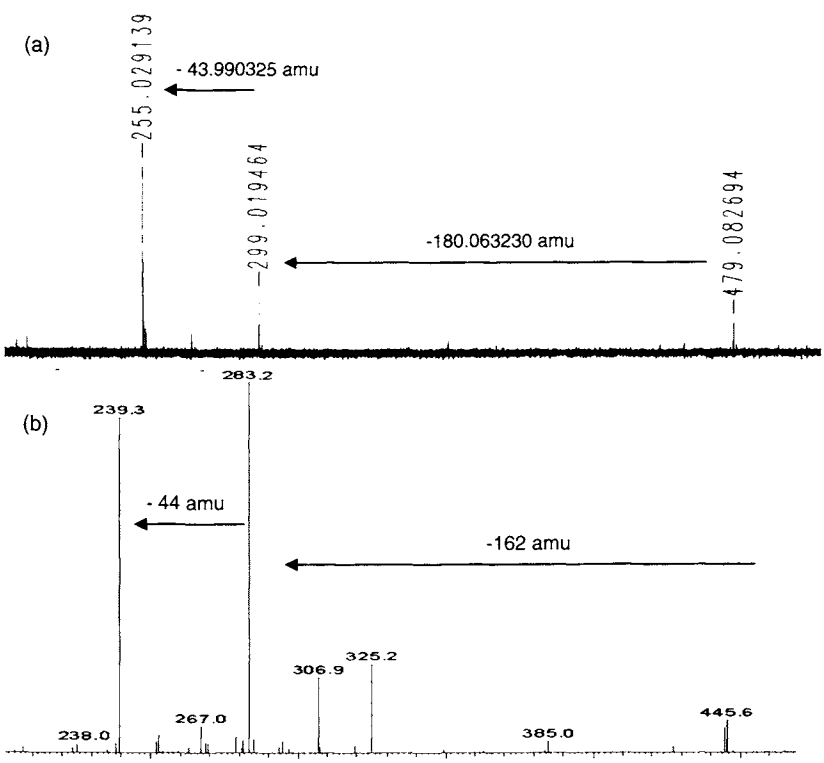
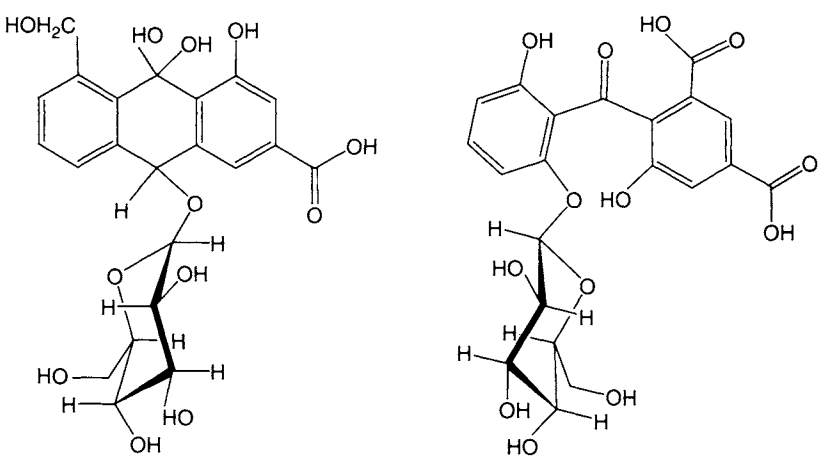


FIGURE 24 (a) Daughter ion mass spectrum of the unknown with nominal mass of 480 Da, acquired using Bruker Apex II 4.7 T FTMS. (b) Daughter ion mass spectrum of a typical impurity, acquired using Finnigan MAT TSQ 7000.

an intense daughter ion was detected at *m/z* of 299.019424. The neutral loss from *m/z* of 479.082594 to 299.019424 corresponded to 180.06323 Da, which agreed very well with the theoretical exact mass of 180.06339 Da for the glucose molecule. The daughter ion mass spectrum acquired using a Finnigan MAT TSQ 7000 mass spectrometer for a typical impurity is given in Figure 24(b). The impurity was used as a control in the H/D exchange experiment. A neutral loss of 162 Da was observed (Figure 24(b)). This is the same neutral loss as for the drug compound and most of its impurities. The loss of 44 Da was observed in both the unknown and the control. Therefore, two possible structures were proposed and are shown in Figure 25.

Structure A contains nine exchangeable protons, while structure B contains eight such protons. A H/D exchange experiment was performed in order to provide the answer. The validity of H/D results was examined by carrying out the H/D exchange for the control compound in a parallel fashion. The control compound contained six exchangeable protons; four of them were the hydroxyl groups in the glucosyl substituant, one was the phenol proton, and the last one was the carboxylic acid proton. A shift in molecular mass of 5 amu was observed when the spectrum was acquired using ESI in the negative ion mode. This demonstrated that the exchange experiment was successful. For the unknown compound a shift of 7 amu was observed, which strongly suggested the presence of eight exchangeable protons rather than nine. It was therefore concluded that



Structure A Structure B

FIGURE 25 **Two proposed structures: structure A with nine exchangeable protons, structure B with eight exchangeable protons.**

structure B was the most likely structure for this unknown impurity. Tables 8 and 9 summarize MS/MS results comparing fragmentation patterns of the D-labeled entities with their H-labeled counterparts. The structure was supported by various 1D and 2D NMR experiments (data not shown). A literature search found that the unknown impurity was recently reported by Terreaux et al.[95] who isolated it from a Tinnevelli Senna pod extract.

IX. SUPPORTING MARKETED DRUG PRODUCTS

A. Continuous Monitoring of Impurity Profile in Drug Product

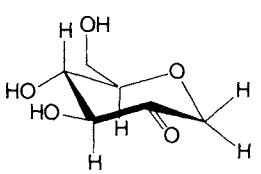
Once an NDA is approved and the drug product is successfully launched, corporate resources shift to production, storage, distribution and marketing of the drug product. The quality and integrity of the drug

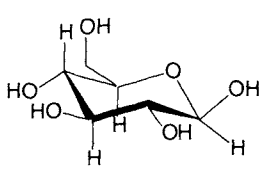
TABLE 8 MS Results of H/D Exchange Experiment: Mass Spectrometer Was Operated at Negative Ion Mode

Controlled compound $[M-H]^-$		Unknown $[M-H]^-$	
In H_2O	In D_2O	In H_2O	In D_2O
445	450	479	486
6 exchangeable protons		8 exchangeable protons	

TABLE 9 MS/MS Results of H/D Exchange Experiment: Mass Spectrometer Was Operated at Negative Ion Mode

Controlled compound		Unknown	
In H_2O	In D_2O	In H_2O	In D_2O
162(445/283)	165(450/285)	180(479/299)	185(486/301)
44(283/239)	44(285/241)	44(299/255)	44(301/257)
The ether bond between glucosyl and phenyl was cleaved; and glucosyl leaves as a tautomer of 2-hydroxyglucal.		The ether bond between glucosyl and phenyl was cleaved; and glucosyl leaves as glucose.	





product is under the increasingly tightened scrutiny of regulatory agencies. Maintaining the standards given in the NDA is the key to protecting the franchise. In case new impurities or degradants are detected during the post-approval stability program, identification and qualification must follow ICH guidelines given in Q3B. A positive identification and quick turn around time help appropriate decision-making. Due to its inherent sensitivity, specificity, and speed, LC/MS is the technology of choice to identify unknown impurities in production batches. New impurities may come from many different sources and may not be related to the drug substance. Cross-contamination due to inadequate cleaning of the manufacturing apparatus, and contaminants due to leachables from packaging components are among the common causes for detection of new impurities. Identification of new impurities in a timely fashion has become a business necessity in defending the drug product franchise. Toxicological studies can only be designed rationally after the structures of impurities are known in order to address issues such as potential toxicity and side effects.

B. Investigation of the Cause for Drug Discoloration

According to the *FDA/Center for Drug Evaluation and Research* the leading causes for drug recall in fiscal year 2001 were:

- Deviations from current good manufacturing practices
- Subpotency
- Failure of stability data to support expiration date
- Failure of drug to dissolve properly
- Correctly labeled product in the wrong carton or package
- Strength of product incorrectly labeled
- Microbial contamination of nonsterile products
- Drug product marketed without an approved new or generic application
- Lack of assurance of sterility in production or testing of sterile drug products
- Discoloration
- Counterfeit dosage form.

Drug discoloration, listed as one of the top 10 causes for drug recall, manifests the stigma that pharmaceutical manufacturers have to tackle with. Discoloration refers to the change or shift in the color on the dosage form from the specified appearance. Discoloration may occur during drug products' transportation, distribution, and/or storage. It can also occur as a result of cross-contamination during the manufacturing process. Discoloration of a drug product is potentially a risk to public health. In cases where discoloration has occurred, the drug product needs to be recalled, and the chemical entity causing discoloration must be identified.

Discoloration, observed as isolated spots on the dosage form, tends to suggest a manufacturing problem such as inadequate cleaning of manufacturing equipment, leading to cross-contamination between different strengths of the same product line, or between different product lines. On the other hand, if discoloration is homogeneous throughout the dosage form, the API and/or excipients must have experienced chemical degradation so that chromophores are altered. Comparison of LC/UV, and/or LC/MS profiles between a normal dosage form with the discolored dosage form may uncover compound(s) responsible for the discoloration. Studies of discoloration, possibly due to the Maillard or browning reaction between lactose and the amine-containing diuretic hydrochlorothiazide, have been reported by Harmon et al.[96] The Maillard reaction may occur, and is always characterized by the formation of brown pigments, when carbonyl compounds are mixed with amines, amino acids, or proteins and are subjected to environmental factors such as high humidity and heat. Diuretic hydrochlorothiazide, after being heated at 60°C for 2 weeks in the presence of lactose and water, was found to form cyclic *N*-substituted glycosylamines as primary condensation products. Blaug and Huang[97] also reported a discoloration problem in a formulation containing dextroamphetamine sulfate and spray-dried lactose. The discoloration was enhanced by the presence of amines and by storage at elevated temperatures. The compound responsible for the discoloration was identified as dextroamphetamine-hydroxymethylfurfural, a reaction product between the active pharmaceutical ingredient and lactose.

Quinidine polygalacturonate (structure illustrated in Figure 26) has been formulated as tablets and is used as an antiarrhythmic agent. The tablet was found to develop a lightly beige color, different from its off-white color, after prolonged storage at ambient conditions. The discoloration was observed in the entire tablet. Chromatographic analysis of

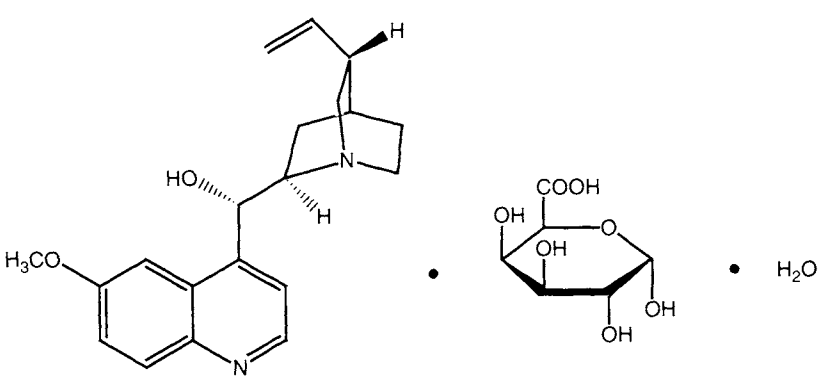


FIGURE 26 Quinidine polygalacturonate.

an extract of the discolored tablets uncovered two late eluting peaks, which were either absent or detected at significantly lower levels in the native tablets whose color was in conformance to specification. The late eluting peaks are shown in Figure 27 at RT of 24.2 and 30.4 min. Mass spectrometric analysis of these two peaks was conducted on a Finnigan MAT TSQ 7000 mass spectrometer equipped with an electrospray interface. Molecular mass for both peaks was determined as 324 Da, which is the same as that of quinidine itself. MS/MS results, shown in Figures 28(a) and (b), suggested that they could be hydroquinidinone and hydroquninone, respectively. These compounds are believed to be oxidation products of hydroquinidine and hydroquinine, which are also process impurities present at low levels in the drug substance. Figure 29 shows the structures for all four compounds. UV spectra comparing quinidine and the hydroqunidinone/hydroquninone are given in Figure 30. The formation of the keto-functionality at a carbon α to an aromatic system resulted in a bathochromic shift in UV absorbance, which is believed to have caused discoloration of the tablets.

C. Investigation of Counterfeit Drugs

Counterfeit drugs pose a serious threat to public health. According to the World Health Organization,[98] a counterfeit medicine is one

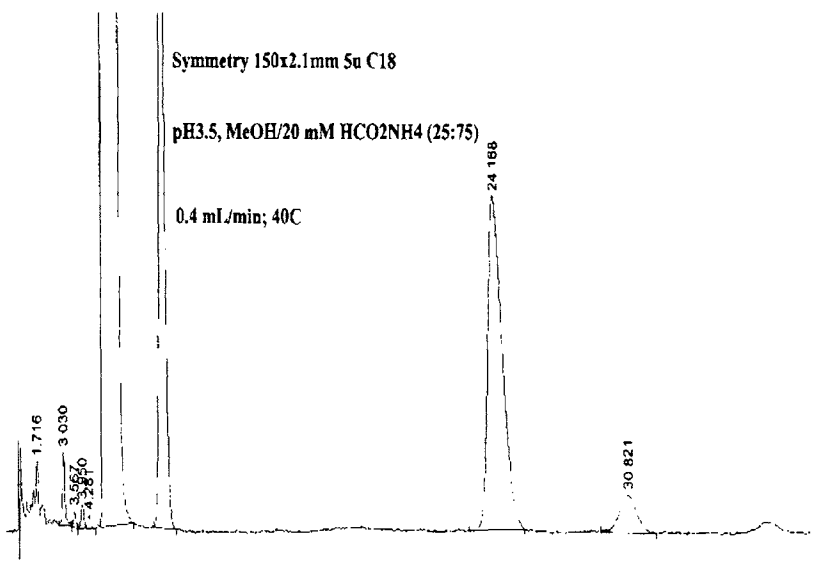


FIGURE 27 LC/UV chromatogram shows two late eluting peaks for the discolored tablets at retention time of 24.2 and 30.4 min.

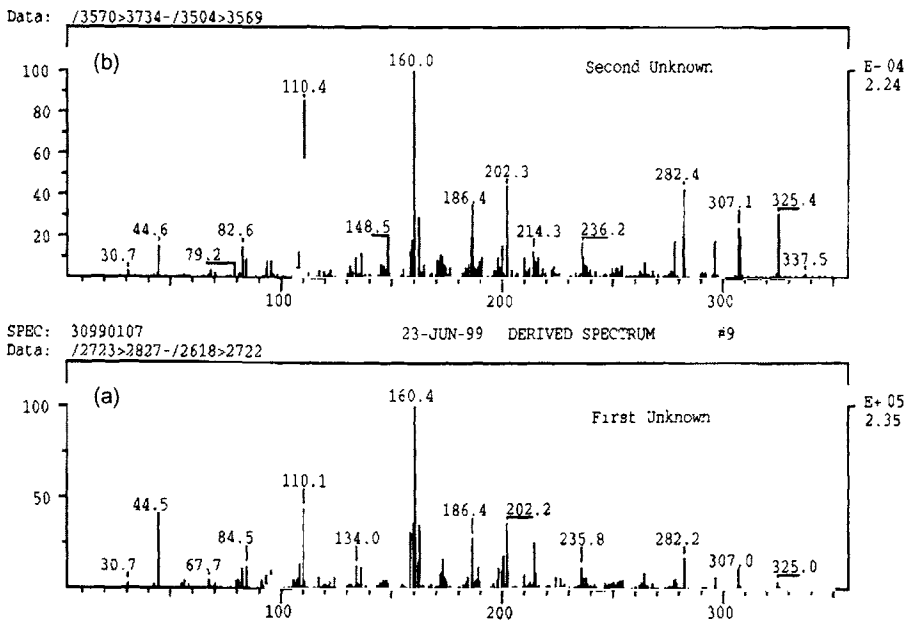


FIGURE 28 MS/MS spectra of the late eluting peaks. Similar fragmentation patterns have been observed for both peaks, indicating that they are likely diastereomers.

"which is deliberately and fraudulently mislabeled with respect to identity and/or source." Counterfeiting can apply to both branded and generic products and counterfeit products may include products with the correct ingredients or with the wrong ingredients, without any active ingredients, with insufficient active ingredients (sub-potent) or with fake packaging." The United States Federal Food, Drug and Cosmetic Act defines a counterfeit drug as "...a drug which, or the container or labeling of which, without authorization, bears the trademark, trade name, or other identifying mark, imprint, or device or any likeness thereof, of a drug manufacturer, processor, packer, or distributor other than the person or persons who in fact manufactured, processed, packed or distributed such drug, and which thereby falsely purports or is represented to be the product of, or to have been packed or distributed by, such other drug manufacturer, processor, packer, or, distributor."[99]

Although there is no universal definition of counterfeit drugs, they are however, deliberately and fraudulently mislabeled with intent to deceive and profit. Counterfeit drugs have rarely been efficacious but are often dangerous and detrimental to public health. Low potency or absence of API altogether will fail to provide the expected and desired therapeutic effect. Others may even have contaminants or adulterants that may be toxic. As the profits from selling a counterfeit drug can be

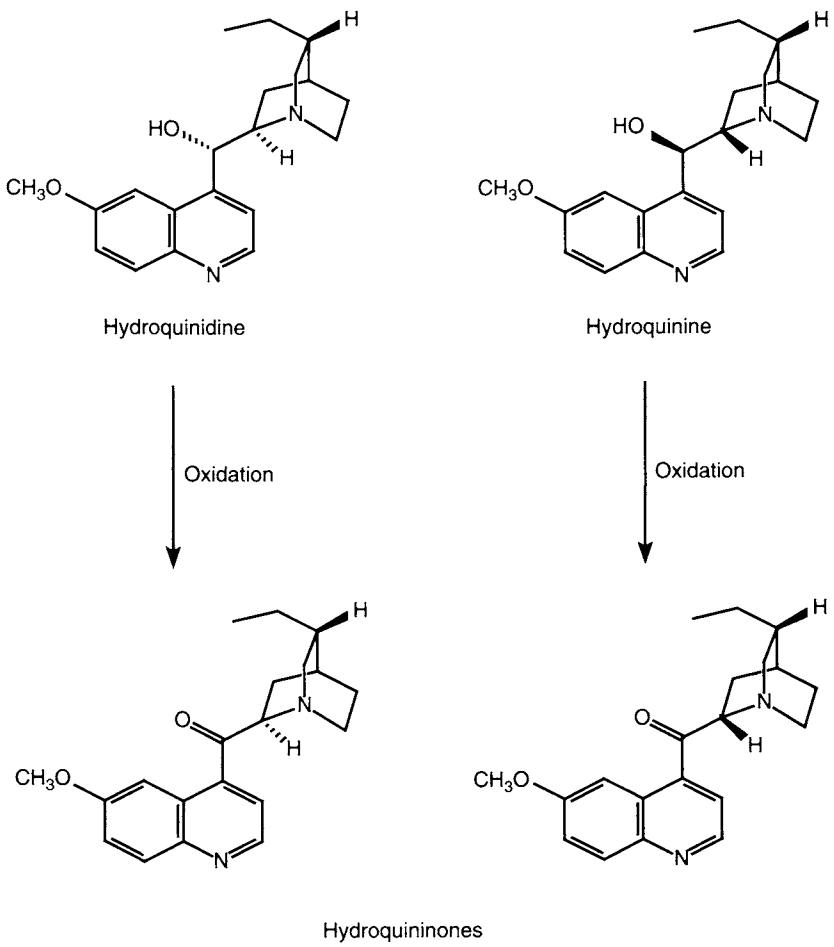


FIGURE 29 **Formation of hydroquinidinone/hydroquininone.**

as high as the sale of illegal narcotics, organized crime has become involved in the "counterfeit drug product industry". Harvey Bale, Director General of the International Federation of Pharmaceutical Manufacturers Associates (IFPMA), said that organized crime faces "less risk in moving into counterfeit medicine than illegal drugs." The World Health Organization and the Pharmaceutical Research & Manufacturers of America estimate that 8–10% of prescription drugs on the world market are counterfeit.[100] Some of the most recent drug recalls due to counterfeiting problem include the following:

Lipitor® recall in Spring 2003—millions of fake pills were smuggled into the United States from abroad. The Lipitor® case highlights the problem of increasing and increasingly sophisticated counterfeit drugs.

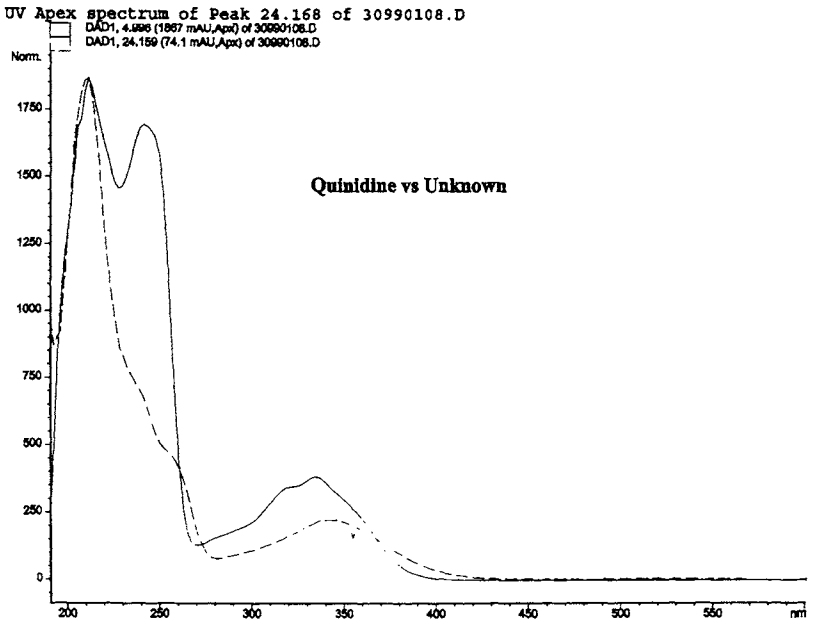


FIGURE 30 **Comparison of UV spectum of quinidine with that of hydroquininoness.**

Glaxo-SmithKline recalled in May 2002 three lots of Combivir® (Lamivudine/zidovudine, used in treatment of HIV infection) due to the fact that it actually contained a drug known as Ziagen® (abacavir sulfate).

Amgen, in May 2001, was forced to recall its Epogen® when counterfeit vials of the product were discovered in the market. Investigation revealed that the counterfeit drug contained only one-twentieth of the labeled strength.

Patients are not the only victims of counterfeit drugs. Pharmaceutical companies can lose income from counterfeit drug competition. Public confidence in the company can be undermined, leading it to stop buying the product and instead purchase a competitor's product even after the counterfeit has been destroyed. A fast identification of the counterfeit drug is in the interest of pharmaceutical companies and the protection of patients' rights.

In July 2002, a DEA regional office confiscated tablets that were counterfeits of a leading brand name prescription analgesic drug. The author's laboratory participated in the investigation. Unlike the identification of drug impurities and degradants, for which a body of knowledge such as the synthetic route, drug product formulation, reactivity of drug compound, etc. is available, the identification of a counterfeit drug molecule begins with no prior information. Identification of counterfeit

drug molecules is akin to "looking for a needle in a haystack." There are hundreds of thousands of organic compounds in the ACS registries. Even drug-related entries in the Merck Index still stand at about 10000. Unarguably, the generation of data using high-resolution mass spectrometry is the best choice in tackling such a challenging task.

Figure 31 shows the ESI/FTMS parent ion mass spectrum of the API in the counterfeit tablet. Inspection of the mass spectrum suggested that this compound contains less than nine carbon atoms because the "A+1" ion intensity is less than 10% of that of monoisotopic peak. The intensity of the "A+2" ion at *m/z* of 340.04810 was estimated at ~13% of the monoisotopic signal. This observation suggested that the counterfeit compound contained some "A+2" element(s) other than Cl and Br. The doublet peaks of the "A+1" isotopic signal at *m/z* of 399.04828 and *m/z* of 399.05638 were observed, with relative intensity at about 1 to 2. This important information led to the supposition that the unknown compound may contain a significant number of atoms from the "A+1" elements such as nitrogen, and/or sulfur. Other common elements in an organic compound may include fluorine and phosphorous. When all the elemental information was built into the search criteria, a list of the top 30 candidates was obtained and is displayed in Table 10. Our procedure for the rapid identification of the best-fit elemental composition can be summarized as follows:

- Build search criteria that include information such as: possible elements and the numbers of each element; maximum and minimum number of double bond equivalency (DBE); maximum tolerance of experimental error in the determined exact mass.
- Examine the DBE value for each candidate from the elemental search report. If a compound shows UV absorbance at a wavelength

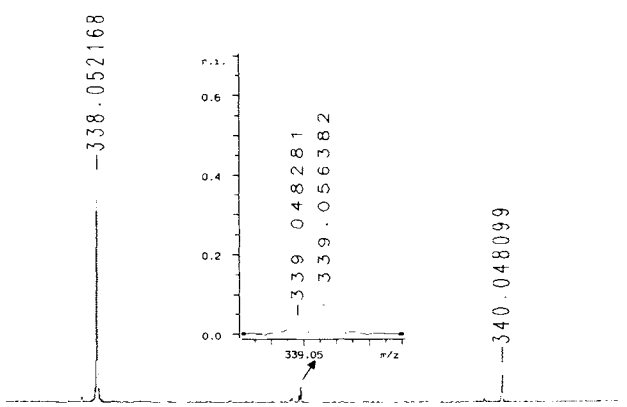


FIGURE 31 FTMS parent ion mass spectrum of the API in the counterfeit tablet.

TABLE 10 Possible Elemental Compositions: Mass Analysis for mass 338.0521680

#	C	H	O	N	S	P	F	Mass	DBE	error
1	7	22	4	3	2	2	0	338.0521470	−0.5	6.203e-08
2	3	12	5	6	0	1	5	338.0521465	−1.0	6.366e-08
3	2	6	0	13	0	1	5	338.0521412	4.5	7.924e-08
4	0	15	2	10	1	2	3	338.0521995	−2.0	9.325e-08
5	10	25	1	0	3	2	1	338.0521301	−1.0	1.120e-07
6	6	15	2	3	1	1	6	338.0521296	−1.5	1.136e-07
7	8	16	2	7	3	0	0	338.0522113	4.5	1.280e-07
8	9	22	7	0	3	0	0	338.0522165	−1.0	1.436e-07
9	7	16	10	2	0	1	1	338.0521109	1.0	1.690e-07
10	6	10	5	9	0	1	1	338.0521056	6.5	1.846e-07
11	9	13	2	6	1	1	2	338.0520887	6.0	2.345e-07
12	5	22	1	6	1	4	0	338.0520775	0.0	2.677e-07
13	1	9	0	14	2	0	3	338.0520638	3.0	2.833e-07
14	8	6	0	6	0	0	8	338.0520713	5.0	2.861e-07
15	2	15	5	7	2	0	3	338.0522690	−2.5	2.989e-07
16	0	12	5	13	2	0	0	338.0520284	1.5	4.131e-07
17	8	19	4	2	0	3	2	338.0520245	1.0	4.246e-07
18	3	15	2	10	3	0	1	338.0520115	1.0	4.630e-07
19	7	7	0	10	0	1	4	338.0523410	8.0	5.118e-07
20	8	13	5	3	0	1	4	338.0523463	2.5	5.274e-07
21	4	9	0	7	0	1	8	338.0523819	0.5	6.327e-07
22	5	16	2	7	1	2	2	338.0523993	1.5	6.843e-07
23	1	7	0	17	0	2	1	338.0524110	7.5	7.187e-07
24	2	13	5	10	0	2	1	338.0524162	2.0	7.343e-07
25	2	15	10	5	0	1	2	338.0519111	−2.5	7.600e-07
26	1	9	5	12	0	1	2	338.0519058	3.0	7.756e-07
27	0	3	0	19	0	1	2	338.0519005	8.5	7.912e-07
28	4	12	2	9	1	1	3	338.0518889	2.5	8.256e-07
29	10	19	2	1	3	0	3	338.0524520	0.5	8.400e-07
30	6	10	0	11	2	0	2	338.0524636	6.5	8.744e-07

higher than 230 nm, then it must bear a reasonable number of DBE. In this case, the compound showed a UV absorbance maximum at 260 nm. Therefore, out of the top 10 candidates, 1, 2, 4, 5, 6, 8, and 9 can be eliminated from consideration because these candidates do not meet the DBE requirement.

- Of the remaining candidates, from top to bottom, generate their theoretical isotopic distributions and compare them to the experimental one. Theoretical isotopic distribution of candidate 3 was an obvious

mismatch because much lower intensities for both the "A+1" and the "A+2" signals were observed. On the other hand, the isotopic fingerprinting of candidate 7 (Figure 32) agreed very well with the pattern seen in Figure 31. Therefore candidate 7 was chosen as the candidate with the best-fit elemental composition.

- The rest of the candidates in the table can be eliminated when the above-mentioned procedure is repeated.

Since the monoisotopic signal is the protonated ion, the elemental composition for the API in the counterfeit tablets was therefore determined as $C_8H_{15}O_2N_7S_3$. A computerized search of The Merck Index using the elemental formula suggested the unknown compound to be Famotidine, an active pharmaceutical ingredient widely used in over-the-counter antiulcerative medicine.

Daughter ion mass spectrum of the compound is shown in Figure 33. A dominant daughter ion was observed at *m/z* of 259.07757. Observation of this ion conformed to the likely fragmentation pattern expected for the Famotidine molecule. Scheme 1 illustrates the fragmentation pathway leading to the formation of this dominant daughter ion.

Confirmative data were obtained by acquiring FTMS and FTMS/MS spectra for a solution made from an authentic commercial product containing Famotidine. Both spectra were identical to those acquired for the counterfeit tablets.

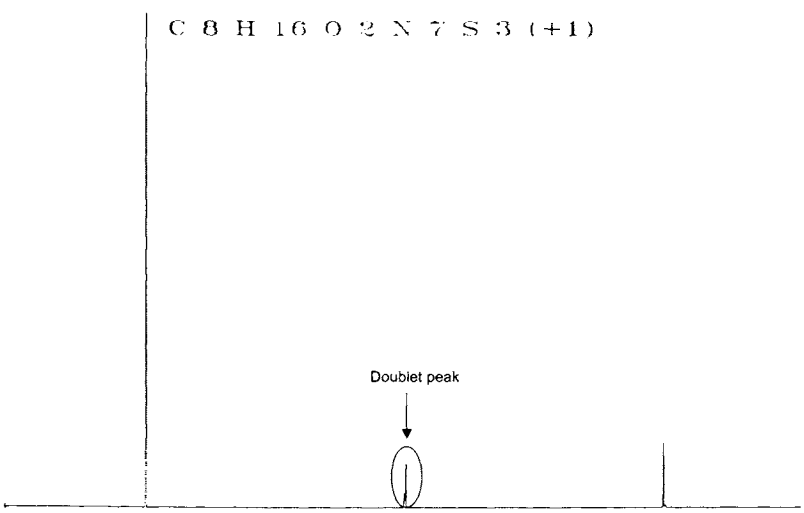


FIGURE 32 **Theoretical isotopic distribution of elemental composition candidate #7.**

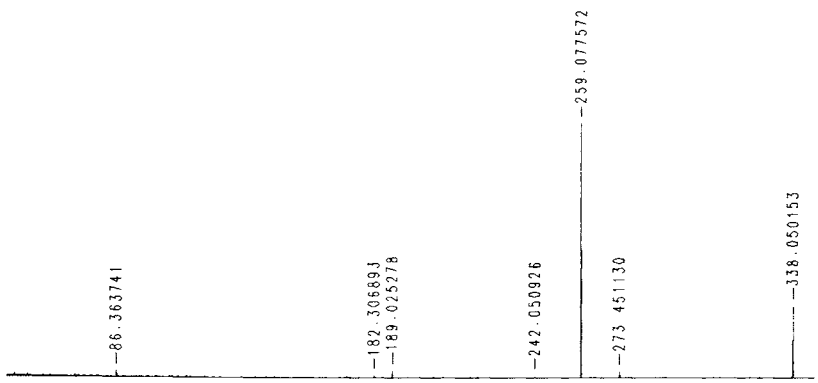
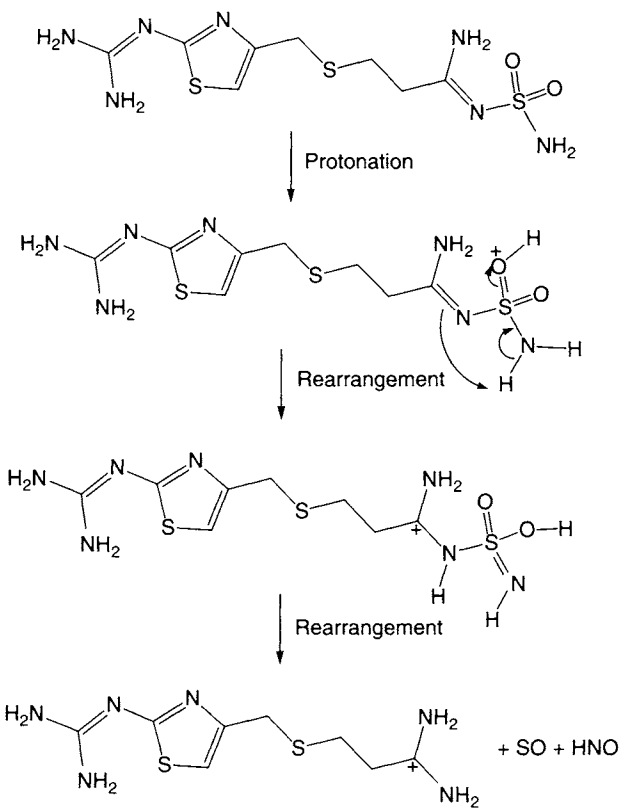


FIGURE 33 **FTMS/MS daughter ion mass spectrum of the API in the counterfeit tablets.**



SCHEME 1 **Fragmentation mechanism leading to the formation of *m/z* of 259.07757.**

D. Patent Protection

Pharmaceutical research and development is a very time-consuming and costly endeavor. The average time required to bring a new molecular entity to market is about 10 years and may cost up to 700 million dollars. Protection of intellectual properties through patent protection is taking high priority in the pharmaceutical industry. Due to its high sensitivity and specificity, LC/MS is a useful tool in the generation of structural information for patent protection of proprietary chemical processes. Drug impurities, especially those specific to the synthetic route can be used as chemical markers to determine whether the synthesis process is being infringed. Almudaris et al.[101] reported an LC-thermospray-MS method used for the identification of three synthetic-route indicative impurities at levels of 50 to 100 ppb (w/w) for the purpose of patent protection. The impurities were extracted and pre-concentrated by normal-phase LC procedures, followed by an on-line structural study by reversed-phase HPLC-TS-MS.

X. CONCLUSION

LC coupling with MS has emerged as one of the most important technologies in pharmaceutical analysis. It is used to ensure the quality and safety of pharmaceutical products. MS can detect molecules in the positive and negative ionization modes, in which molecules are transformed, primarily, into protonated or deprotonated pseudo molecular ions. Due to its inherent analytical capabilities, the technique is widely used in applications ranging from monitoring, identifying, and characterizing drug impurities to investigating deficit mass balance, drug product discoloration, and drug product counterfeiting. The obvious benefit of using LC/MS is the opportunity it affords for rapid identification of unknown compounds by generating compound specific information such as molecular weight and elemental composition. Substructural information can also be generated from CID experiments. Many other benefits can also be realized, such as increased gain in confidence and decreased time required for HPLC method development. Using a "generic HPLC/MS method," data can be collected in a consistent manner throughout the development life cycle of a pharmaceutical product.

However, LC/MS is not a panacea. In mass spectrometry, molecules must exist as ionic species in order to be detected. The ionization efficiency can vary over many orders of magnitude depending on the structure of the compounds studied. For nonpolar and neutral drug compounds, an ESI or APCI interface may not be sensitive enough to allow meaningful structure determination. Although the assignment of various signals in an ESI/MS mass spectrum is usually straightforward,

artifacts can arise from electrochemical reactions during the electrospray ionization process. Thermally induced degradation is also a common phenomenon with both ESI and APCI interfaces due to the heat input to aid in the evaporation of solvents. The presence of artifacts will obviously complicate accurate determination of the molecular weight for trace level drug impurities. Knowledge of the sample in terms of its formulation, packaging materials, synthetic route, chemical activity of drug compound, etc. is always beneficial in the identification of unknown impurities.

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